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Sinclair, Adriane Watkins
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Pask, Andrew
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Flutamide-induced hypospadias in rats: A critical assessment

Adriane Watkins Sinclair[a], Mei Cao[a], Andrew Pask[b], Laurence Baskin[a], Gerald R. Cunha[a,*]

[a] Department of Urology, University of California San Francisco, 400 Parnassus Avenue, Box A610, San Francisco, CA 94143, USA
[b] Department of Anatomy and Neuroscience, School of BioSciences, University of Melbourne, Melbourne, Victoria 3010, Australia

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ABSTRACT

This paper provides the first detailed description of flutamide-induced hypospadias in the rat based upon wholemount, histologic, three-dimensional reconstruction, scanning electron microscopic, and immunocytochemical analysis. The penile malformations elicited by this potent anti-androgen include a substantial proximal shift in the urethral meatus that clearly conforms to the definition of hypospadias based upon specific morphological criteria for this malformation. Through examination of the normal penile development and flutamide-induced abnormal penile development observed in prenatally oil- and flutamide-treated rats, our analysis provides insights into the morphogenetic mechanism of development of hypospadias. In this regard, a common theme in normal penile development is midline fusion of epithelia followed by removal of the epithelial seam and establishment of midline mesenchymal confluence during development of the penile urethra and prepuce, processes which are impaired as a result of prenatal flutamide treatment. The developmental processes occurring in normal penile development, through comparison with development of female external genitalia and those impaired due to prenatal flutamide treatment, are consistent with critical role of androgen receptors in normal penile development in the rat, and the specific penile abnormalities embodied in flutamide-induced rat hypospadias.

1. Introduction

Hypospadias is a common urogenital anomaly in boys occurring in 1:200 to 1:300 male births (Baskin, 2000), and the incidence of this congenital defect in the USA has increased over the last few decades (Paulozzi et al., 1997; Paulozzi, 1999). The etiology of hypospadias for the majority of patients remains unknown but is considered to be the combined effects of genetic susceptibility and environmental exposure to endocrine-disrupting compounds (Baskin and Ebbers, 2006; Willingham and Baskin, 2007; Wang and Baskin, 2008; Kalfa et al., 2011).

Hypospadias is a penile malformation primarily defined by proximal displacement of the urethral meatus from the tip of the glans penis to mid-shaft or into the perineum (Cunha et al., 2015). Associated with the defect in the urethral meatus is (a) absence or hypoplasia of the corpus spongiosum near the abnormal urethral orifice, (b) absence of the ventral aspect of the prepuce, and (c) chordee (abnormal curvature of the penis) (Baskin et al., 1998). Rodent hypospadias differs substantially from human hypospadias, perhaps secondary to species differences in penile anatomy, penile development and endocrinology of the human versus the mouse and rat (Cunha et al., 2015).

In a general sense, hypospadias represents a perturbation of patterning of the elements constituting the penis, especially the positioning of the urethral meatus. While the various forms of human hypospadias are obvious on physical examination, hypospadias is subtle in the mouse. Mouse hypospadias induced by endocrine disruptors does not involve “mid-shaft” or perineal malformations similar to those in humans. The reason for this is that most of the mouse penile urethra forms by direct canalization of the embryonic urethral plate (Seifert et al., 2008; Hynes and Fraher, 2004b). However, in mice and rats the urethral plate does not extend into the distal aspect of the developing penis (Hynes and Fraher, 2004a, b; Sinclair et al., 2016b, 2016a; Kluth et al., 2011), and thus a different morphogenetic mechanism is utilized to generate the distal aspect of the penile urethra in mice. In the developing human penis, the urethral plate canalizes to form a widely open urethral groove, whose edges (urethral folds) fuse in the ventral midline to form the tubular penile urethra (Li et al., 2015b; Shen et al., 2016). In mice a ventral groove (the preputial-urethral groove) forms in the distal aspect of the developing mouse penis independent of and distal to the urethral

Abbreviations: MUMP, male urogenital mating protuberance; MCC, MUMP corpora cavernosa; CCG, corpus cavernosum glandis; CCUr, corpora cavernosa urethrae; SEM, scanning electron microscopy; AR, androgen receptor; 3DR, three dimension reconstruction; FM, furrowed mucosa; CPM, central penile mesenchyme

* Corresponding author.
E-mail address: gerald.cunha@ucsf.edu (G.R. Cunha).

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plate. Subsequent epithelial fusion within the preputial-urethral groove (similar to that seen in human penile development) results in formation of the distal aspect of the mouse penile urethra and the ventral aspect of the external prepuce (Sinclair et al., 2016b). As will be shown in this paper, similar developmental processes occur in the developing rat penis. Thus, malformations of the distal aspect of the mouse and rat glans penis may be particularly relevant models for human hypospadias as both animal models involve perturbation of distal fusion events.

A major difference between of the penis of humans versus rats and mice is the terminology of anatomical parts. In humans the penile shaft (also called the body) and glans constitute a pendulous external projection of the body. In contrast, in rats and mice the penile body lies deep to the body surface, while the so-called elongated glans projects from the body surface. In the flaccid state the rat and mouse glans penis is hidden within the external prepuce, a prominent hair-bearing elevation in the rodent perineum that does not have an analogous structure in humans (Sinclair et al., 2016c, 2016b). In contrast, the glans penis of both rats and mice has an integral internal prepuce homologous to the human prepuce (Blaschko et al., 2013; Sinclair et al., 2016c). Skeletal elements of the rat glans penis (like that of the mouse) consist of an os penis proximally and the distal fibrocartilagenous male urogenital mating protuberance (MUMP) (Beresford and Burkart, 1977; Izumi et al., 2000; Cunha et al., 2015). The MUMP of the mouse is a prominent distal projection at the tip of the glans penis, while that of the rat is completely hidden by the encircling internal prepuce (Cunha et al., 2015).

Erectile bodies are consistent penile elements in mice, humans and rats. A corporal body has been described for all three species although its localization is profoundly different in rodents versus humans. In humans, the corporal body resides within the pendulous penile shaft (body). In contrast, in rats and mice the corporal body is located in the penile body which lies deep to the body surface (Cunha et al., 2015). Humans have a corpus spongiosum surrounding the penile urethra, which is homologous with the mouse corpora cavernosa urethrae located ventral to the penile urethra. The mouse has two additional erectile bodies within the glans, namely the corpus cavernosum glandis and MUMP corpora cavernosa (Cunha et al., 2015). The rat glans penis also contains a corpus cavernosum glandis (Murakami, 1984), but the presence or absence of the corpora cavernosa urethrae and MUMP corpora cavernosa have not been previously described (Cunha et al., 2015). Additionally, the morphologic complexity of the glans penis and urethral meatus has been inadequately described in the rat, and may be substantially different in rats versus mice. A detailed understanding of the anatomical differences between the rat and mouse glans penis may reveal which species is the best animal model for human hypospadias.

Development of the rat penis, similar to that of the mouse, begins prenatally and is completed postnatally. By contrast, human penile development occurs exclusively during prenatal periods, and is complete by 17–18 weeks gestation (Li et al., 2015b). As in the mouse, postnatal fusion events are likely to be involved in the development of the rat urethral meatus. However, inadequate literature on rat penile development has hindered understanding of hormonally induced rat hypospadias.

Hypospadias in the rat has been induced by prenatal treatment with various compounds. A PubMed search of "rat" in the Title/Abstract and "hypospadias" in All Fields resulted in 41 relevant articles (review articles excluded). Hypospadias in the rat has been induced by phthalates, which are known to inhibit androgen production by the testes (Hu et al., 2009): Di (*n*-butyl) phthalate (Zhang et al., 2011; Zhu et al., 2009; Jiang et al., 2007; Mylchreest et al., 1999, 1998; Wolf et al., 1993; Li et al., 2015a; Liu et al., 2012), di-isobutyl phthalate (Saillenfait et al., 2008), di-isooctyl phthalate (Saillenfait et al., 2013), di-(2-ethylhexyl) phthalate (Li et al., 2013; Wolf et al., 1999), di-n-hexyl phthalate (Saillenfait et al., 2009) and by phthalate mixture (Gray et al., 2000). Hypospadias has also been reported in rats treated prenatally with the estrogen, diethylstilbestrol (Vorherr et al., 1979), an effect that in part may be due to reduction in androgen production by the testes (Goyal et al., 2007). Other agents that induce hypospadias in rats include the 5α-reductase inhibitor, finasteride (Bowman et al., 2003; Clark et al., 1993, 1990; Anderson and Clark, 1990), androgen receptor antagonists such as flutamide (Pallares et al., 2014; Welsh et al., 2008; Foster and Harris, 2005; Uda et al., 2004; McIntyre et al., 2001; Zakaria et al., 2000; Mylchreest et al., 1999), vinclozolin (Schneider et al., 2011; Gray et al., 1999, 1994), procymidone (Ostby et al., 1999; Wolf et al., 1999), alone or in combination (Christiansen et al., 2008), and p,p′-DDE (Wolf et al., 1999). Other mixtures containing vinclozolin, procymidone, linuron, prochloraz, benzyl butyl phthalate, dibutyl phthalate and diethylhexyl phthalate also induce hypospadias in rats (Rider et al., 2008). Prochloraz, a fungicide that inhibits aromatase activity, inhibits androgen synthesis and antagonizes the androgen receptor, is also effective in inducing hypospadias in rats (Noriega et al., 2005), as are testicular enzyme inhibitors of steroid 17alpha-hydroxylase and C 17–20 lyase (Goldman et al., 1976; Bloch et al., 1971; Goldman and Kenneck, 1970).

The central problem with the above hypospadias studies in rat is that this defect has been inadequately described. Not a single paper displays histological images of adult rat hypospadias. Of the 41 primary papers of rat hypospadias only 8 papers include histological images: 3 showing fetal penile histology and 5 showing prepubertal penile histology between postnatal days 7–30 (Anderson and Clark, 1990; Zhang et al., 2011; Jiang et al., 2007; Welsh et al., 2008; Zhu et al., 2009; Li et al., 2013). The majority of papers report rat hypospadias in the text only, or provide unclear gross images of external perineal anatomy, making it impossible to distinguish preputial abnormalities from penile abnormalities.

The aim of this paper is to (a) comprehensively define normal anatomy of the rat glans penis for the first time, (b) to precisely define hypospadias in the rat induced by the androgen receptor antagonist, flutamide, (c) to investigate the morphogenetic mechanism of formation of rat hypospadias, and (d) to describe and discuss the expression of androgen receptor protein (AR) in relation to the malformations elicited by the anti-androgen, flutamide. In so doing, we reveal for the first time the similarities and differences between human, mouse and rat hypospadias and assess the potential relevance of the rat as an animal model of human hypospadias.

2. Materials and methods

2.1. Animals

Animal care and research protocols were approved by the Animal Care and Use Committee of the University of California, San Francisco (UCSF). Adult Sprague Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, USA) and their offspring were housed in polycarbonate cages (20×25×47 cm^3) with laboratory grade pellet bedding in the UCSF Pathogen Specific Barrier facility. Rats were given water ad libitum and fed LabDiet 5058 (PMI Nutrition International, P. O. Box 66812, St. Louis, MO 63166). This study is based upon 31 male and 26 female prenatally oil-treated and 34 male prenatally flutamide-treated rats.

2.2. Hormonal treatments

Seven pregnant Sprague Dawley dams were weighed and injected subcutaneously once daily from gestational day 14–20 with flutamide dissolved in sesame oil (Sigma-Aldrich) at a concentration of 25 mg/kg body weight. Six control group dams were injected with sesame oil. The effectiveness of prenatal flutamide in impairing androgen action was verified by its effect in reducing anogenital distance in male rats (Fig. 1).

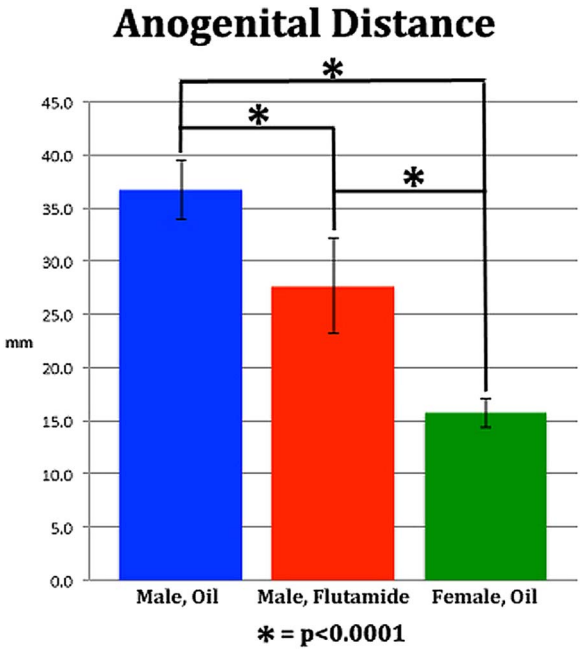

Fig. 1. Graph of anogenital distance in adult (60 d old) Sprague-Dawley rats treated prenatally with oil or flutamide. *= $p < 0.0001$.

2.3. Specimen preparation and analysis

Flutamide or oil-treated Sprague Dawley rats were euthanized at birth and at the 10 and 60 days postnatal. Sex was confirmed by gonadal inspection. External genitalia were dissected and fixed in 10% buffered formalin for a minimum of 24 h. Seven micrometer thick sections were stained with hematoxylin and eosin as described previously (Sinclair et al., 2016a). Anogenital distance (AGD) was measured from photographs.

2.4. Scanning electron microscopy

Surface details of adult rat glans penis and the external prepuce of rats aged 0, 10 & 60 days were elucidated using scanning electron microscopy (SEM) as described previously (Blaschko et al., 2013).

2.5. Three dimensional reconstruction (3DR)

Anatomical three-dimensional computer reconstructions were created from serial transverse sections utilizing SURF driver 3.5 software (SURF driver, University of Hawaii and University of Alberta). Sections were digitized to achieve linear tracings of relevant structures, including glans penis, clitoris, os penis, urethra, corpus cavernosum, and prepuce. Digital linear tracings from adjacent sections were serially aligned using Photoshop software (Adobe, Inc. San Rafael, CA 94903) and then exported into SURF driver for three-dimensional reconstruction.

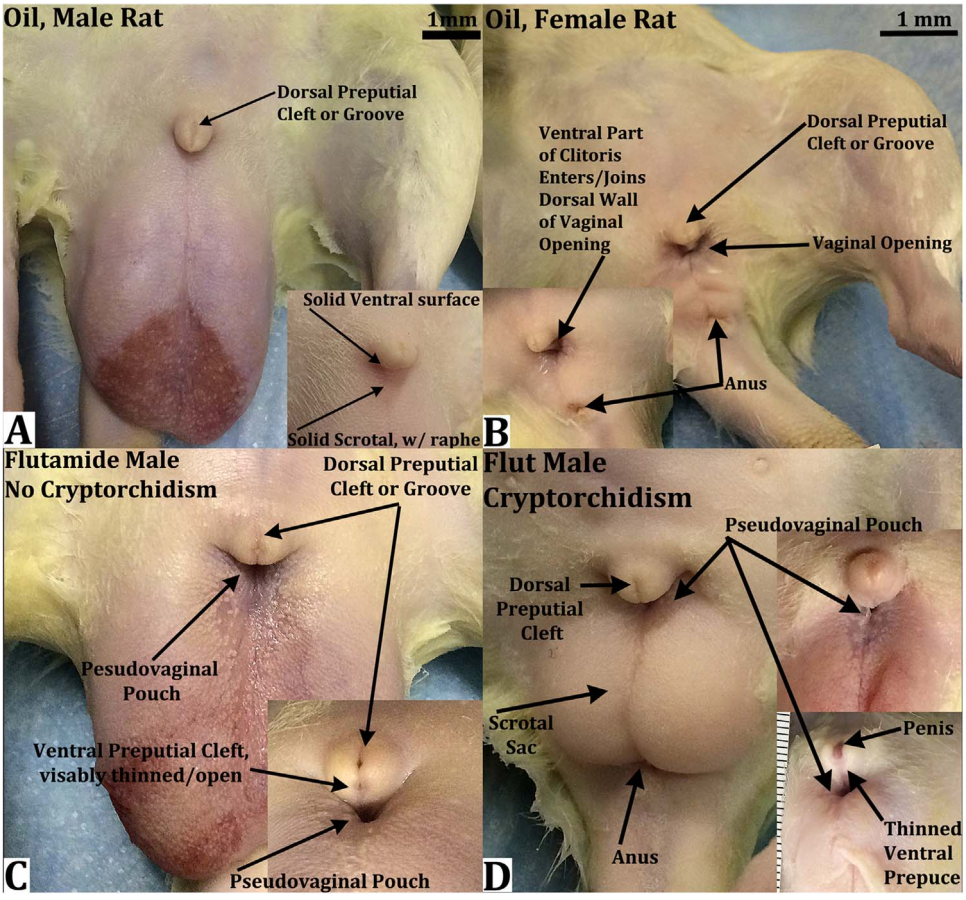

Fig. 2. External genitalia of adult rats. (A) oil-treated male, (B) oil-treated female, (C) prenatally flutamide-treated male without cryptorchidism, and (D) prenatally flutamide-treated male with cryptorchidism. A dorsal preputial cleft or groove is visible in the external prepuce of all oil males and females and prenatally flutamide-treated males. A ventral preputial cleft or thinning of the ventral surface of the external prepuce is present in all flutamide-treated males as well as a pseudovaginal pouch. The inset in (D) shows the tip of the penis, which normally would not be visible. For all images cranial is toward the top of the figure.

2.6. Morphometric analysis

Metrics of pertinent key penile morphological features such as glans penis length or cartilage length were measured by counting the number of 7 μm serial histologic sections from the distal penile tip to the structure of interest as described previously (Schlomer et al., 2013).

2.7. Immunohistochemical analysis

Rat penile specimens were formalin fixed, paraffin embedded and serially sectioned at 7 μm. Immunohistochemistry was carried out as previously described (Rodriguez et al., 2012b) utilizing anti-androgen receptor (rabbit monoclonal, diluted 1:200, GTX 62599, Genetex, Irving, CA). Signal detection was achieved using the Vector ABC System (Vector Laboratories, Foster City, CA, USA) followed by exposure to diaminobenzidine (Sigma®). Sections exposed to all steps except the application of the primary antibodies were used as negative controls.

3. Results

3.1. Normal adult rat penile anatomy

The elevation in the perineum of 60-D-old adult normal male and female rats is the prepuce and not the glans penis or clitoris, which are both "internal" organs lying deep to the prepuce. The hair-bearing male external prepuce has a dorsal cleft or groove, a preputial meatus and a subtle midline ventral raphe (Figs. 2A & 3A) (ventral surface=closest to the anus). The adult rat penis consists of a so-called body and glans that are joined at the right angle bend (Fig. 4A) (Goyal et al., 1998). The glans penis is housed within the space created by the external prepuce, whose inner lining reflects onto the surface of the glans penis near the glans/body junction (at the right angle bend) (Figs. 4A & 5, green arrows). The rat also has an internal prepuce, which is integral to the glans penis (Figs. 4A, 5 & 6). Fig. 4A, in which the external prepuce of a prenatally oil-treated rat has been partially removed, demonstrates the considerable distance between the blunt tip of the glans penis and the external preputial meatus (Fig. 4A, blue arrow). The surface of the glans penis is adorned with penile spines (Figs. 5, 6) as described previously (Aronson and Cooper, 1967; Orr and Brennan, 2016). The dorsal penile surface is unremarkable except for the presence of penile spines (Fig. 6C). The glans penis has a prominent ventral raphe and subtle lateral grooves (Fig. 6A, D–E). "End-on" views reveal a furrowed mucosa (FM) and an associated dorsal blunt process at 12 o'clock (Fig. 6A), which can also be seen in histological sections (Fig. 7A & B). The actual urethral meatus is obscured by the FM (Fig. 6A). When the internal prepuce is removed by dissection, the extent of the FM is revealed, and the Y-shaped urethral meatus is seen (Fig. 6B) with the cartilaginous male urogenital mating protuberance (MUMP) projecting into the Y-shaped urethral meatus from its mid-dorsal position

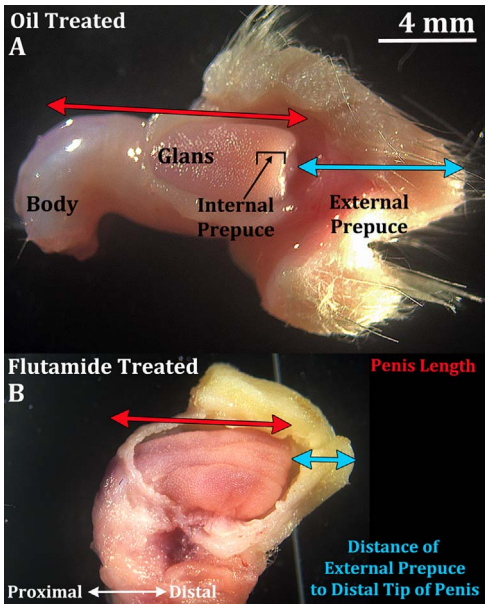

Fig. 4. Gross images of the penis and external prepuce of adult rats with part of the external prepuce removed by dissection. (A) oil-treated male and (B) prenatally flutamide-treated male. Note that the blunt-tipped penis is located within the external preputial space. Length of the glans penis (red arrows) is reduced in the flutamide-treated specimen (B). Distance from the tip of the external prepuce to the distal tip of the penis is also reduced in prenatally flutamide-treated male rats **(blue arrow)**. In A, the oil-treated male, the junction of the body of the penis ends and the glans begins at the right angle bend as described previously (Goyal et al., 2004). The external prepuce and the position of the internal prepuce are indicated. Addition images of the internal prepuce are seen in Figs. 5 and 6.

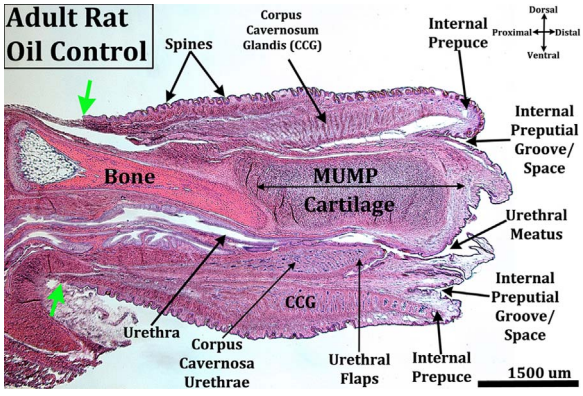

Fig. 5. Mid-sagittal section of the adult prenatally oil-treated rat penis stained with hematoxylin and eosin. Anatomical structures of interest are labeled. Green arrows denote the point where the inner lining of the external prepuce (removed) reflects onto the surface of the glans penis near the glans/body junction. Distal is to the right, dorsal to the top of the figure.

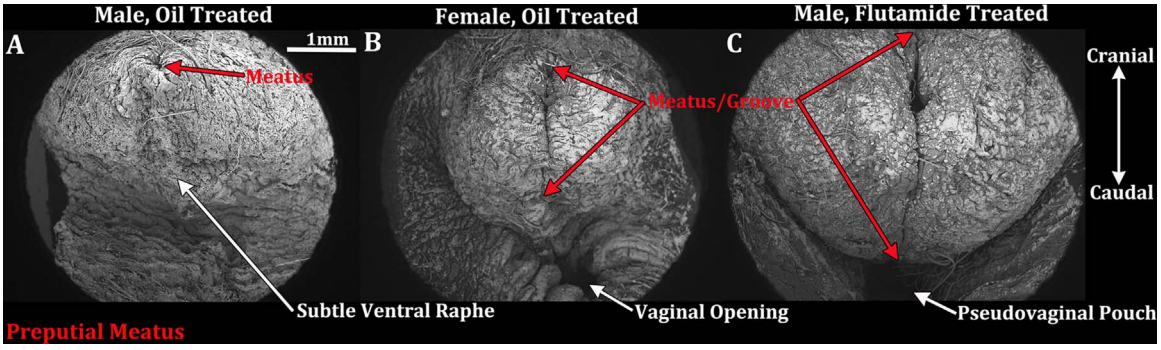

Fig. 3. Scanning electron micrographs of the external prepuce of adult rats. (A) oil-treated male, (B) oil-treated female, and (C) prenatally flutamide-treated male. The size of the preputial meatus is defined in red with the flutamide-treated male having the longest preputial meatus/groove for males. For all images cranial is toward the top of the figure.

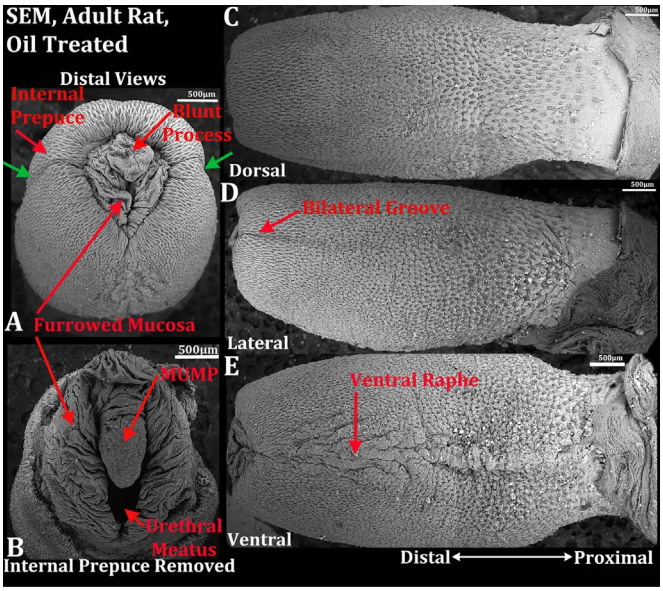

Fig. 6. Scanning electron micrographs of adult prenatally oil-treated rat penis. (A) end on view of distal penile tip, (B) distal penile tip with internal prepuce removed by dissection, (C) dorsal view, (D) lateral view, and (E) ventral view. Green arrows in (A) denote the bilateral grooves in the internal prepuce. The penile surface texture seen in (A, C–E) indicates the abundance of penile spines.

(Figs. 6B, 7D–F). The distal surface of the MUMP is finely textured and not heavily furrowed in contrast to the FM (Fig. 6B).

The anatomical relationships between external morphology and internal penile structures were revealed by histology and three-dimensional reconstruction (3DR). Fig. 5 is a mid-sagittal section of the glans penis from its blunt distal tip to the proximal region where the inner lining of the external prepuce reflects onto the surface of the glans penis near the glans/body junction (Fig. 5, green arrows). Dorsal to the urethra is the cartilaginous MUMP, which dorsally overlaps the os penis (Figs. 5, 7 [lateral view] & 8). There are several erectile bodies within the rat glans penis: (a) the circumferential corpus cavernosum glandis located distally within the internal prepuce (CCG in Figs. 5 & 7E-G), (b) the bilateral MUMP corpora cavernosa (MCC in Fig. 7F), and (c) the corpora cavernosa urethrae (CCUr in Figs. 5, 7G).

The internal prepuce is composed of 3 components: 1 dorsal and 2 lateral (Figs. 7C–D & 8C). Distally the 3 rudiments of the internal prepuce are distinct (Figs. 7C–D, 8C), but proximally they are fused together (Fig. 7E-G). Bilateral grooves seen in SEMs (Fig. 6A & D) and shallow grooves in transverse histological images (Fig. 7D–E) are adult manifestations of the fusion events involved in formation of the internal prepuce. The encircling internal prepuce surrounds the FM and covers the urethral meatus (Figs. 5–8). Accordingly, the urethral meatus opens into the internal preputial space (Fig. 6) near the distal tip of the glans penis (Fig. 7 [ventral view] & 8). Projecting into the ventral aspect of the urethral lumen are the urethral flaps, which contain erectile tissue forming the corpora cavernosa urethrae ventral to the urethra (Figs. 5, 7F & G, 8B & D).

The MUMP of the rat is composed of cartilage, and the distal aspect of the MUMP is covered ventral-laterally with epithelium (Figs. 5 & 7D–E). The MUMP cartilage extends proximally for nearly half of the length of the glans penis and dorsally overlaps the distal tip of the os penis (Figs. 5, 7–8). The os penis is located dorsal to the urethra in the proximal half of the glans penis (Figs. 5, 7–8).

3.2. Abnormal adult penile anatomy in prenatally flutamide-treated rats

Prenatal flutamide treatment caused cryptorchidism (n=8), which may be bilateral (n=2) or unilateral (n=6), or had no effect on testicular descent (n=4) when examined at 60 days postpartum (Fig. 2C–D). Males treated prenatally with flutamide have a blind ending pseudovaginal pouch, irrespective of the presence or absence of cryptorchidism (Fig. 2C & D). Prenatally flutamide-treated rats have a prominent elongated preputial opening or groove that continues onto the ventral surface of the external prepuce similar to that of females (Fig. 3). In some rats the external prepuce is defective (partially absent), and thus the glans penis is exposed and clearly visible externally (Fig. 2D, inset).

Partial removal of the external prepuce demonstrates two important effects of prenatal flutamide treatment: (a) considerable shortening in length of the glans penis, and (b) truncation of the external prepuce (Fig. 4B). Surface morphology and histology of prenatally flutamide-treated adult male rats are highly abnormal in so far as the glans penis is fused (tethered) ventrally to the inner wall of the external prepuce (Figs. 9A & C, 10F–G and 14H). Pronounced bilateral bulges protrude laterally, and are separated from the dorsum of the glans penis by deep bilateral grooves (Figs. 9–10). Viewed end-on, the 3 rudiments constituting the internal prepuce are present, but the 2 lateral rudiments do not extend to their normal fusion in the ventral midline (Figs. 9A, 10D–G). The furrowed mucosa seen in oil-treated rats (Fig. 6A) is less pronounced in prenatally flutamide-treated adult rats (Fig. 9A). The distinct dorsal blunt process associated with furrowed mucosa is absent (compare Figs. 6 and 9), and the MUMP (normally hidden by the internal prepuce) is visible in prenatally flutamide-treated adult rats (Fig. 9A).

The anatomical relationships in adult prenatally flutamide-treated rats between external and internal penile morphology were investigated using histology and three-dimensional reconstruction (3DR). The abnormal internal prepuce (Figs. 9A & 10) partially surrounds the "furrowed mucosa" (FM), which stains intensely with H & E in flutamide-treated rats and lightly in oil-treated rats (Compare Figs. 7B & 10C). Proceeding proximally into the serial section set, the 3 rudiments of the internal prepuce are present dorsally and laterally (Fig. 10B–D) and eventually coalesce to form the internal prepuce, which remains incomplete ventrally (Figs. 10D–G & 14H). Fusion of the dorsal and lateral rudiments of the internal prepuce is manifested by deep external grooves and prominent bilateral surface bulges (Figs. 9, 10E–F, & 11B, 14H).

The erectile bodies within the glans penis are poorly developed, defective or absent in prenatally flutamide-treated adult rats. The corpus cavernosum glandis never extends to the ventral midline (Figs. 10F–G, 14H). The bilateral MUMP corpora cavernosa lying lateral to the MUMP cartilage are hypoplastic (Compare Figs. 7F & 10E–F). The urethral flaps are devoid of erectile tissue, and their proximal extensions as corpora cavernosa urethrae are absent (Figs. 10 & 14H).

Relative to the length of the glans penis, it appears that the MUMP cartilage is longer than its normal counterpart in prenatally flutamide-treated adult rats (Compare Figs. 8 and 11). However, absolute length of the MUMP cartilage in oil-treated adult male rats was 3262 µm (St. Dev= ± 372, n=4), while in adult prenatally flutamide-treated rats MUMP cartilage length was reduced to 2187 µm (St. Dev = ± 623, n=4). However, total length of the glans penis in flutamide-treated males was only 3087 µm (St. Dev= ± 703) compared to 7248 µm in oil-treated rats (St. Dev = ± 843). Thus, the MUMP cartilage in the flutamide-treated rat is 67–75% of total glans length compared ~45%, of total glans length in oil-treated rats. The MUMP cartilage dorsally overlaps the os penis for a short distance in prenatally flutamide-treated rats, as is the case for oil-treated rats (Figs. 7–8 and 10–11).

Perhaps the most striking malformation observed in prenatally flutamide-treated adult rats is ventral tethering of penile tissue to the inner surface of the external prepuce. The resultant bilateral ventral tethers are observed along the entire proximal half of the glans penis (Figs. 9, 11A & I). Attachment of the right and left tethers to the inner surface of the external prepuce defines a tubular space that constitutes

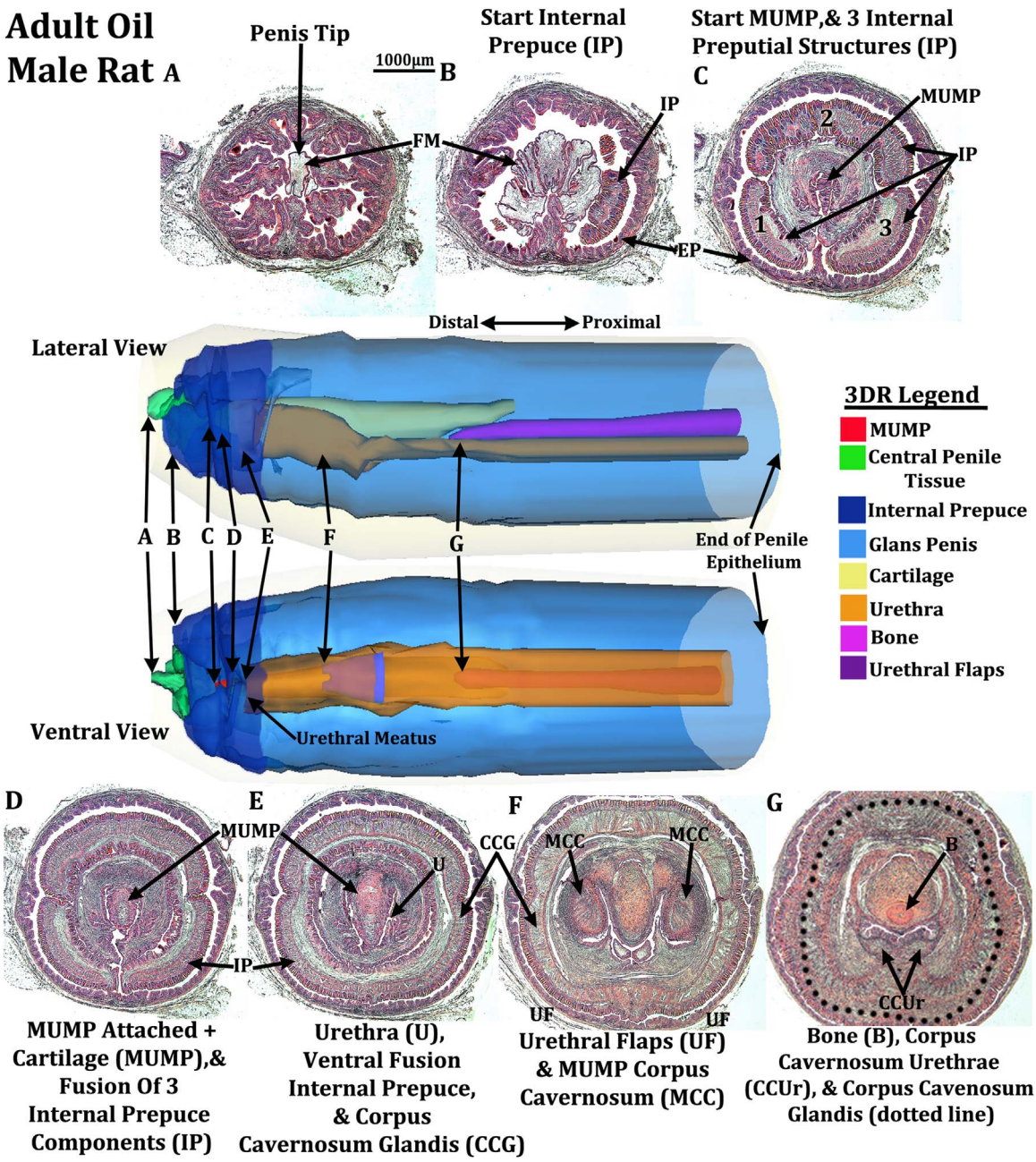

Fig. 7. Transverse sections stained with hematoxylin and eosin (A–G) of the adult prenatally oil-treated rat penis. Sections start from the distal tip of the penis (A) and proceed to (G) the proximal appearance of the os penis bone (labeled B). Locations of the transverse sections are mapped onto the lateral and ventral views of the three dimensional reconstructions (3DR) of the adult prenatally oil-treated rat penis. Note transverse section (E) is at the urethral meatus. 3DR legend identifies the colored 3DR structures. IP = internal prepuce, EP = external prepuce, MUMP = male urogenital mating protuberance, CCG = corpus cavernosum glandis, U = urethra, UF = urethral flaps, MCC = MUMP corpora cavernosa, B = bone, FM = furrowed mucosa and CCUr = corpora cavernosa urethrae.

part of the course for urine. This abnormal channel is not a proper penile urethra because this channel is not completely surrounded by penile tissue (Figs. 10F–G & 14H). Instead, the ventral portion of this channel is located within the wall of the external prepuce (Figs. 10F–G & 14H). The critical feature and definition of a penile urethra is its location within and completely surrounded by penile tissue. The channel seen in Figs. 10F–G and 14H does not meet this definition. However, for simplicity the opening of this abnormal channel into the internal preputial space will be called the urethral meatus, which is abnormally located near the midpoint of the glans penis of flutamide-treated rats (Figs. 10–11). Accordingly, the urethral meatus of flutamide-treated rats is located 2,013 µm (St. Dev= ± 397, n=4) from the distal penile tip, as opposed to its normal distal location at 642 µm (St. Dev= ± 68, n=4) from the distal penile tip (Figs. 7–8). These data confirm that the urethral meatus is near the glans mid-point in flutamide-treated rats versus at the distal penile tip in controls. Another malformation of the “urethra” of prenatally flutamide-treated rats occurs where the urethra angles ventrally (Fig. 10, lateral view & G & Fig. 11F–H). Here the “urethra” divides into 3 tubes (U in Fig. 10G): (a) the 2 dorsal tubes end blindly; (b) the ventral tube is the “urethra” conveying urine from the bladder (Figs. 10G & 11F–H). Table 1 lists the morphological differences observed in penises of prenatally oil- and flutamide-treated rats.

3.3. Normal adult clitoral anatomy

The hair-bearing perineal elevation of prenatally oil-treated adult female rats is not the clitoris, but instead is female prepuce as

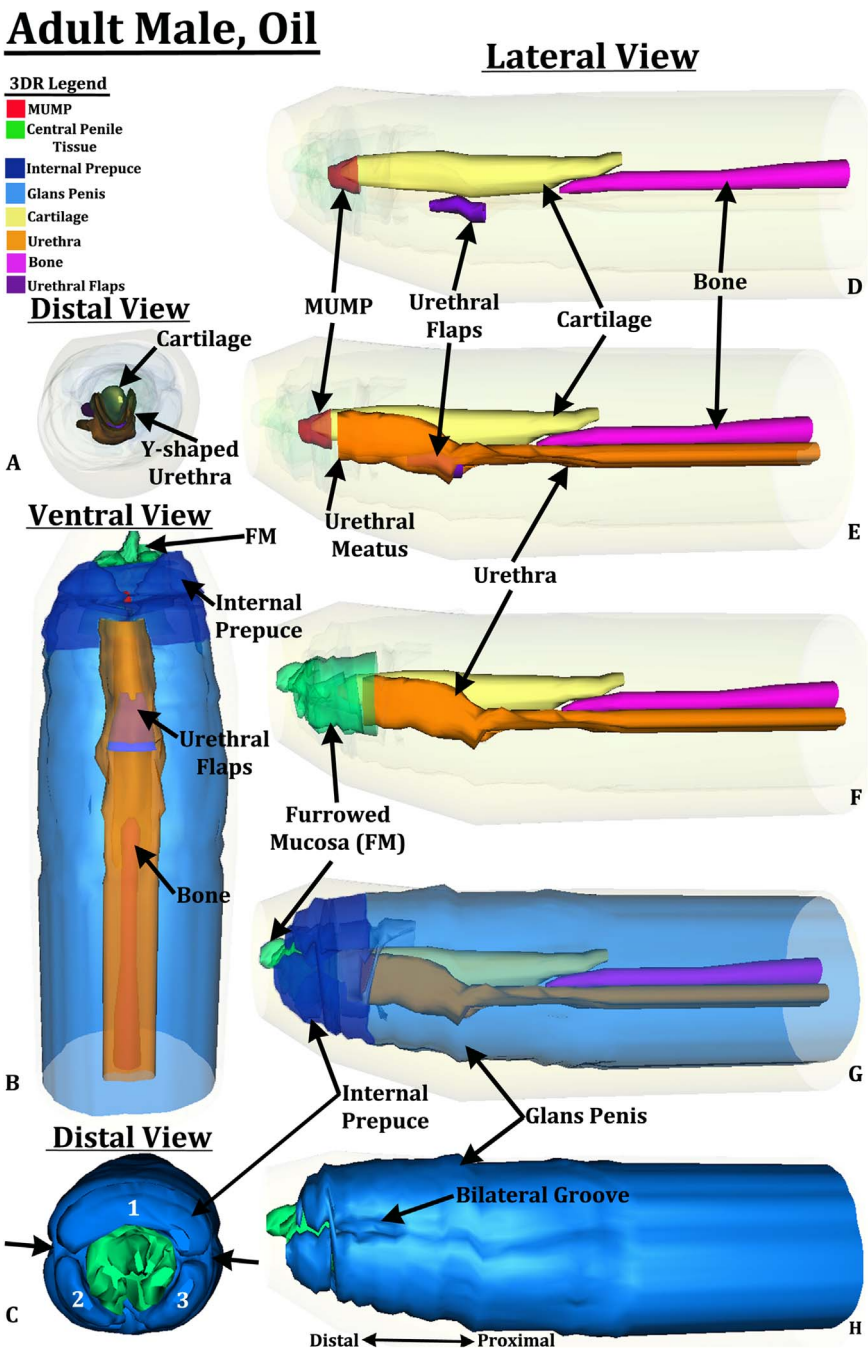

Fig. 8. 3DR of the adult prenatally oil-treated rat penis. (A) Distal view of 3DR with internal prepuce and glans penis rendered semi-transparent, (B) ventral view with the external surface of the glans penis rendered semi-transparent, (C) distal view showing the components of the internal prepuce opaque, (D-H) lateral views with progressively added structures to highlight the positions, sizes, and shapes of internal penile structures. In (C) black arrows depict the bilateral grooves in the internal prepuce. The 3 distal components of the internal prepuce are numbered in white.

described previously (Sinclair et al., 2016c). An extensive cleft or groove extends along the dorsal and ventral surfaces of the female prepuce to end near the vaginal opening (ventral= the surface closest to the anus, same designation as in the male) (Figs. 2B & 3B). This cleft in the prepuce is the preputial meatus. However, since this meatus transmits urine, the term, urethral meatus, is also an acceptable term. The actual rat clitoris, like that of the mouse (Weiss et al., 2012), is situated internally and is defined by the inverted U-shaped clitoral epithelial lamina which is open ventrally (Fig. 14I, double-headed arrows). Thus, stroma "circumscribed" by the U-shaped clitoral epithelial lamina is continuous with stroma ventral to the clitoris, and thus the rat clitoris is tethered ventrally and thus immobile. The position of the urethra relative to the clitoral epithelial lamina varies on a proximal to distal basis. Proximally, the female urethra lies mostly below the U-shaped clitoral epithelial lamina (Fig. 14I). More distally, the female urethra is mostly situated within the confines of the clitoral epithelial lamina (not illustrated) as is the case for mice (Weiss et al., 2012).

3.4. Development of external genitalia of prenatally oil- and flutamide-treated male and female rats

Given the many penile malformations observed in prenatally flutamide-treated rats, developmental studies were undertaken to gain insight into normal development of the rat penis and flutamide-induced penile malformations.

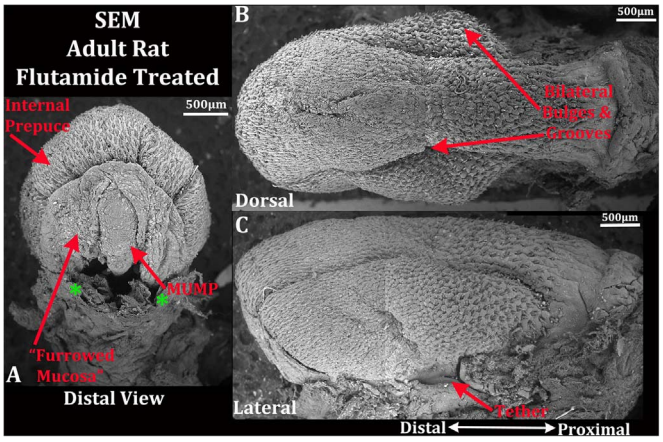

Fig. 9. Scanning electron micrographs of adult prenatally flutamide-treated rat penis. (A) distal tip, (B) dorsal view, and (C) lateral view. Green asterisks in (A) denote the locations of the 2 ventral tethers attaching the ventral penile tissue to the external prepuce.

3.4.1. Developmental anatomy at postnatal day 0

The glans penis and clitoris develop from the embryonic genital tubercle. At birth (Fig. 12) the genital tubercles of males and females form a perineal prominence covered externally by a hair-bearing epidermis. The first row of Fig. 12 depicts histologic sections through the distal aspect of genital tubercles of prenatally oil-treated newborn male and female rats and prenatally flutamide-treated male rats. The numbers associated with each image are the number of serial sections from the distal tip of the genital tubercle to the numbered section. In the first row (Fig. 12A–C) all 3 treatment groups exhibit similar morphology. Near the distal tip of the genital tubercle, a hair-bearing preputial epidermis surrounds the light staining mesenchyme of the prepuce in which preputial gland ducts (PPGD) are seen (Fig. 12A–B), or in the case of the oil-treated female are beginning to invade into the preputial mesenchyme (Fig. 12C). In all 3 treatment groups, a prominent preputial-urethral groove is present (Fig. 12A–C, green asterisks). In males this central epithelium of the preputial-urethral groove circumscribes the central penile mesenchyme (CPM in Fig. 12A & B), destined to form the stroma of the glans penis.

The second row of Fig. 12 contains sections taken at more proximal positions (note section numbers). All 3 treatment groups appear similar at this proximal-distal level (Fig. 12 D–F). The salient events at this level are: (a) expansion of the epithelial circumscribed central penile mesenchyme (CPM), (b) formation of distinct mesenchymal condensations in dorsal and ventral-lateral positions within the central penile mesenchyme (Fig. 12D–F). (c) The epithelium circumscribing the central penile mesenchyme in the male specimens (Fig. 12 D–E) can now be called the external preputial lamina (PPL), which eventually will delaminate to create the external preputial space. The penile mesenchyme circumscribed by the external preputial lamina is characterized by intense H & E staining and contrasts with the lightly stained mesenchyme of the external prepuce (Double-headed red arrows in Fig. 12 D–E). The female equivalent of a preputial lamina is present in Fig. 12F. Note the continued presence of the preputial-urethral groove in all 3 treatment groups (green asterisks in Fig. 12 D–E).

The third row of Fig. 12 (Fig. 12 G–I) contains sections of newborn male and female genital tubercles taken at more proximal positions (note section numbers). In the glans penis of prenatally oil- and flutamide-treated rats, ventral mesenchymal columns (VC) are surrounded by epithelium (Fig. 12G–H). Such ventral mesenchymal columns are not seen in the female specimen (Fig. 12I). Note the continued presence of the preputial-urethral groove in all 3 treatment groups. However, in the case of the oil-treated male, an epithelial fusion event has occurred, which has divided the original preputial-urethral groove into a tubular urethra dorsally (UR in Fig. 12G), which distinguishes the oil-treated male specimen from the other treatment groups. Note that the epithelium defining the urethra in the oil-treated male (Fig. 12G) is continuous with the epithelium of the preputial lamina.

The fourth row of Fig. 12 contains sections taken at more proximal positions (note section numbers). This row (Fig. 12 J–L) illustrates two important points. (a) Mesenchymal confluence has been established between the epithelium-circumscribed ventral mesenchymal columns and central penile mesenchyme in both oil-treated males and prenatally flutamide-treated males (double-headed black arrows in Fig. 12J–K). (b) In oil-treated males right-left ventral mesenchymal confluence within the prepuce (Fig. 12J, red double headed arrow) has been established so that now all penile tissues are circumscribed by the "stand alone" preputial lamina without epithelial attachments to the preputial epidermis (skin). In prenatally flutamide-treated males (Fig. 12K & N), the open preputial-urethral groove persists just like the female (Fig. 12L–O), and the epithelium of the preputial lamina is continuous with the hair-bearing preputial epidermis (Fig. 12K). The preputial lamina is discontinuous dorsally in the oil-treated females at this level (Fig. 12L), but complete in oil-treated males and prenatally flutamide-treated males (Fig. 12J–K). Note that the epithelium defining the urethra in the oil-treated male (Fig. 12J) remains continuous with the epithelium of the preputial lamina.

The fifth row of Fig. 12 contains sections taken at more proximal positions (note section numbers). The striking difference in the oil-treated male (Fig. 12M) is a "stand alone" tubular penile urethra completely surrounded by penile stroma, due to establishment of right-left mesenchymal confluence ventral to the urethra (black double-headed arrow in Fig. 12 M) and the absence of the preputial-urethral groove due to its right-left fusion. In both oil-treated females and prenatally flutamide-treated males the preputial-urethral groove persists (Fig. 12N–O, green asterisks), and thus at this level a tubular urethra is absent. At this level the preputial laminas of all 3 treatment groups are distinctly different. In prenatally oil-treated males, the preputial lamina is 360 degrees complete except for a very narrow gap in the ventral midline (Figs. 12M, 14A, 15D & E, & 16). In prenatally flutamide-treated males bilateral gaps in the preputial lamina are present on both sides of the ventral groove, and thus broad ventral mesenchymal confluence is observed between the penile and preputial mesenchymas (Fig. 12N, red double-headed arrows), which are the basis of ventral tethering in adult prenatally flutamide-treated males (Figs. 9A & 14H). In prenatally oil-treated females, the preputial lamina is discontinuous dorsally (Fig. 12O) as described above. Bone (os penis), MUMP cartilage, or differentiated erectile bodies are not present in treated or untreated males and females at postnatal day 0.

3.4.2. Developmental anatomy at postnatal day 10

The skip from day 0 to day 10 postnatal involves many developmental changes. However, examination of serial sections at day 10 is most edifying. Again, we begin with distal sections and proceed proximally. Since clitoral development is so vastly different from penile development, oil- and flutamide-treated penile development will be described together, with clitoral development following.

In the first row of Fig. 13 the penile urethra and central penile mesenchyme can be seen, and the lateral mesenchymal columns are just beginning to appear as mesenchymal cores surrounded by epithelium (Fig. 13 A & B). Initially development of the lateral mesenchymal columns is similar in oil- and flutamide-treated rats (Fig. 13A–B & D–E) in so far as the epithelium initially surrounds the lateral mesenchymal columns (Fig. 13B & D-E), but subsequently the epithelium disappears in more proximal sections as the mesenchyme of the ventral columns becomes confluent with the central penile mesenchyme (Fig. 13 G–H). Differentiation of the lateral mesenchymal columns into the MUMP corpora cavernosa (MCC) is advanced in oil-treated rats (Fig. 13G & J) and less so in flutamide-treated rats

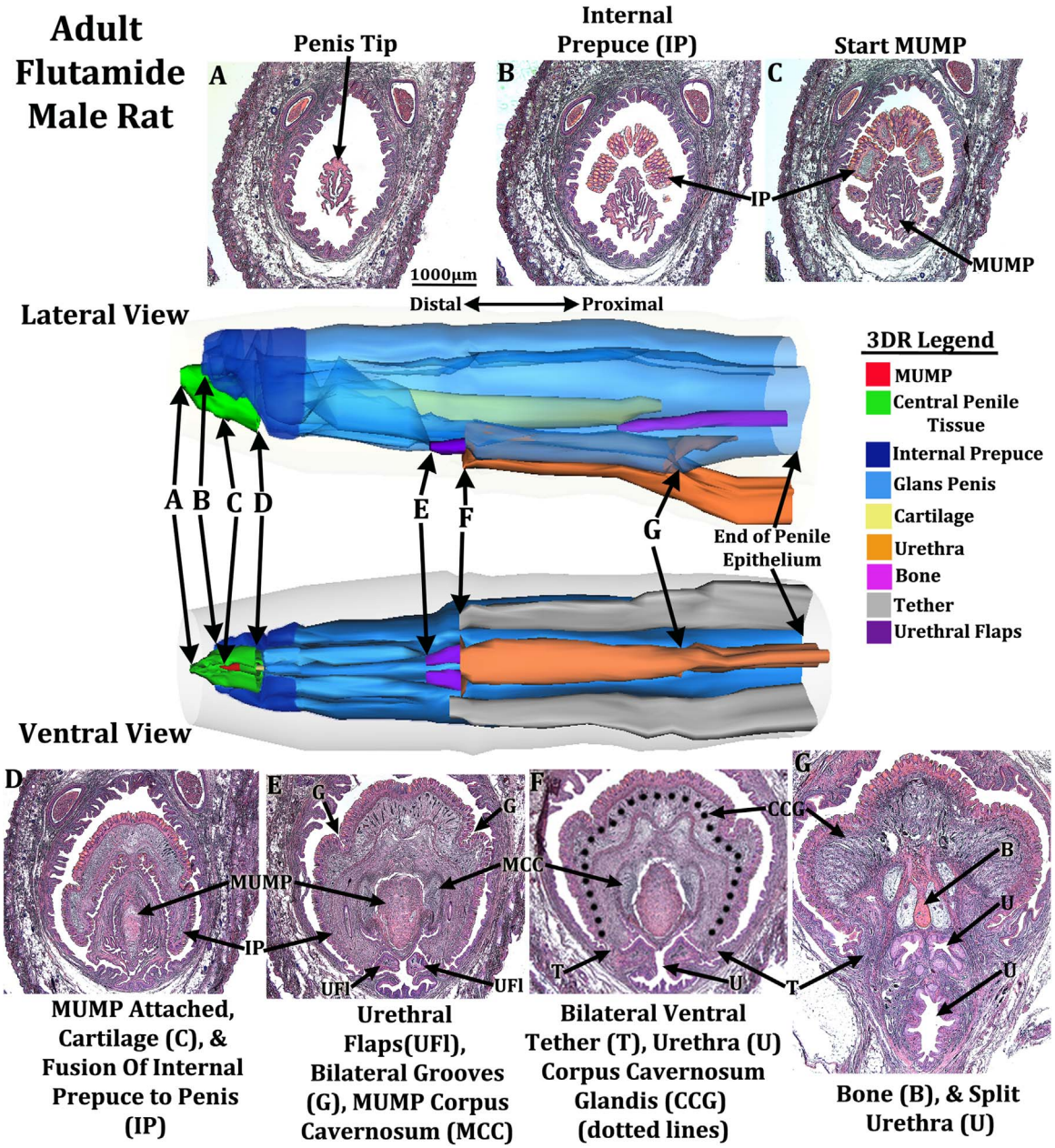

Fig. 10. Transverse penile sections stained with hematoxylin and eosin (A–G) of the adult prenatally flutamide-treated rat penis. Sections start from the distal tip of the penis (A) and proceed proximally to the appearance of the os penis in (labeled B in section G). Locations of the transverse sections are mapped onto the lateral and ventral views of the 3DRs. 3DR legend identifies the colored 3DR structures. IP = internal prepuce, MUMP = male urogenital mating protuberance, G = bilateral grooves in internal prepuce, UFl = urethral flaps, MCC = MUMP corpora cavernosa, U = urethra, T= tether, CCG = corpus cavernosum glandis, and B = bone.

(Fig. 13 H & K) even though the overall developmental process of the lateral column development is similar in prenatally oil- and flutamide-treated rats.

The rudiments of the internal prepuce appear in the oil-treated glans penis in an orderly fashion with the appearance of the dorsal and bilateral ventral-lateral rudiments, initially surrounded/demarcated by epithelium (Fig. 13G). Fusion of the dorsal and the 2 ventral-lateral rudiments of the internal prepuce is seen in Fig. 13J, and the fusion events are marked externally by 3 shallow grooves: (a) one in the ventral midline and (b) two in lateral positions (Fig. 13J). All 3 shallow grooves are represented in adulthood as raphes/grooves (Fig. 6). During the fusion process, mesenchymal confluence is established between all three rudiments of the internal prepuce (double-headed red and black arrows in Fig. 13J) in oil-treated specimens. Mesenchymal condensations within the 3 rudiments of the internal prepuce are the precursors of the corpus cavernosum glandis (CCG) that is well differentiated in Fig. 13M. At this serial section level (Fig. 13J) the oil-treated glans penis is surrounded by the external preputial lamina (later destined to delaminate to form the external preputial space). Immediately deep to the external preputial lamina is the mesenchyme of the internal prepuce defined on its inner aspect by the internal preputial lamina (also destined to delaminate) to form the internal preputial space (Fig. 13J). Fig. 13M, a proximal section, depicts the “stand alone” penile urethra (which is no longer continuous with other epithelia) (compare Fig. 13A, D, G, J, M). The MUMP cartilage is present, well differentiated in Fig. 13D, G, J, M and dorsally overlaps the os penis (Fig. 13M). The ventral mesenchymal columns have differentiated into the corpora cavernosa urethrae (Fig. 13J & M, & 14 G). In summary, epithelial-circumscribed paired lateral and ventral mesenchymal columns as well as the internal preputial rudiments appear in a distal to proximal sequence and fuse with the central penile mesenchyme, while the urethral epithelium separates from

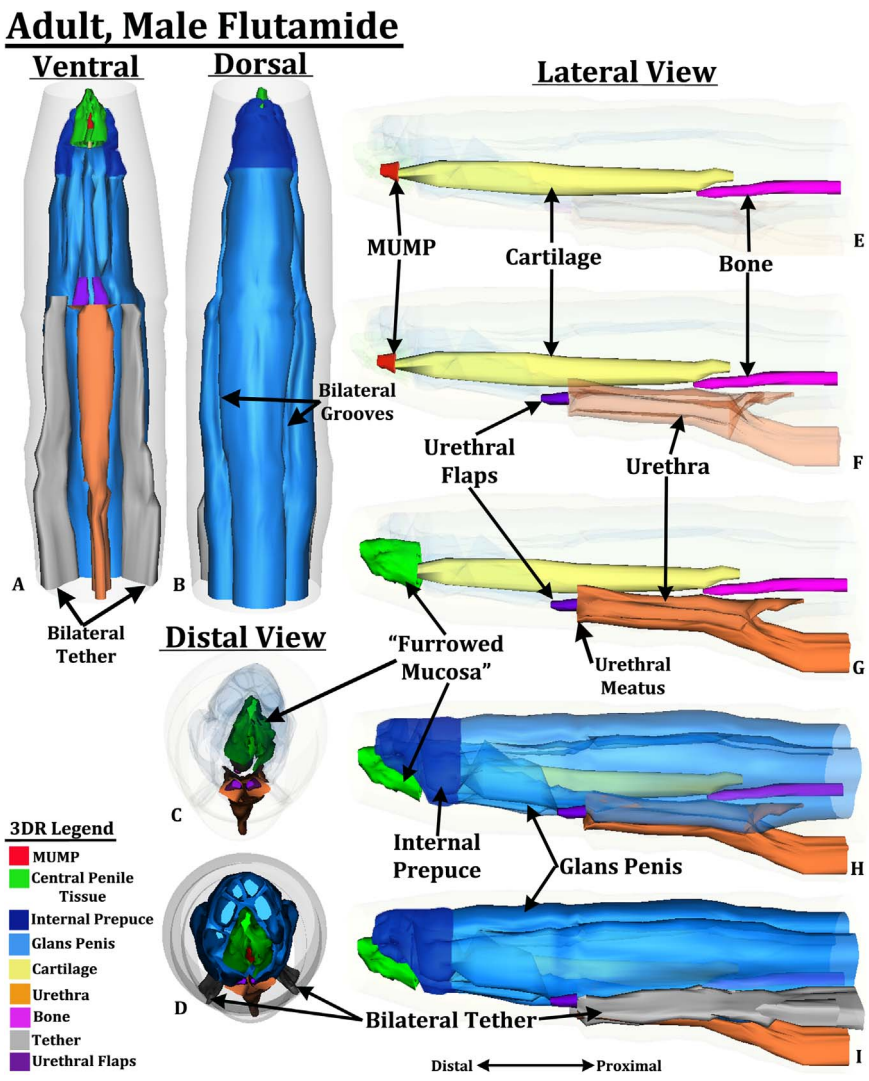

Fig. 11. Three dimensional reconstruction (3DR) of the adult prenatally flutamide-treated rat penis. (A) Ventral view of the 3DR with all structures opaque, (B) dorsal view all structures opaque, (C) distal view with internal prepuce and glans penis rendered semi-transparent, (D) distal view all structures opaque, (E–I) lateral views with progressively added structures to highlight the positions, sizes, and shapes of the internal penile structures.

surrounding epithelia to form the "stand alone" urethra in prenatally oil-treated rats.

Penile development in prenatally flutamide-treated males is highly abnormal, in part due to the overall truncation in the length of the prenatally flutamide-treated glans penis (Fig. 4). Differentiation of erectile bodies is abnormal. For example, lateral mesenchymal columns (normally destined to form the MUMP corpora cavernosa, MCC) as well as the rudiments of internal prepuce (normally destined to form the corpus cavernosum glandis) are unexpectedly present in the same section (Fig. 13B), implying a perturbation in proximal-distal patterning of internal penile rudiments. The lateral mesenchymal columns of prenatally flutamide-treated males fuse normally with the central penile mesenchyme, but differentiate into hypoplastic MUMP corpora cavernosa (MCC in Fig. 13E & H). Ventral mesenchymal columns seen in Fig. 13E fail to undergo development of corpora cavernosa urethrae (compare Fig. 13D, G, J, M with Fig. 13E, H, K, N and also 14 G & H). The three rudiments of the internal prepuce appear as expected in flutamide-treated specimens, and fusion occurs between the single dorsal and the paired lateral rudiments (Fig. 13E, H, K, N). However, ventral midline fusion of the paired lateral rudiments does not occur (Fig. 13 K & N). The corpus cavernosum glandis associated with the internal preputial lamina is hypoplastic and defective ventrally (Fig. 13N), and the stroma "circumscribed" by the U-shaped preputial lamina is confluent with ventral stroma in flutamide-treated specimens, as is the case for oil-treated females (Fig. 13N & O, red arrows). MUMP cartilage and bone are observed in prenatally flutamide-treated males.

The "penile" urethra of prenatally flutamide-treated rats is also abnormal and resembles that of the female. In both prenatally flutamide-treated males and oil-treated females (not illustrated), the urethra angles ventrally in proximal sections (Figs. 10 and 11). At this point of angulation, dorsal urethral extensions are observed in both prenatally flutamide-treated males (Figs. 10 and 11) and oil-treated females (not illustrated). Accordingly, sections at this level show 2 or 3 "urethras" (Fig. 13N & O). The dorsal "urethral extensions" end blindly, while the ventral channel is the definitive urethra conveying urine from the bladder (Figs. 10, 11, 13N & O). In summary, the glans penis of prenatally flutamide-treated rats simultaneously exhibits certain masculine and feminine features.

Given that male and female external genitalia develop from the genital tubercle, certain aspects of clitoral development in oil-treated females at day 10 are shared in common with penile development even though the relative positions of anatomical structures are different. For instance, during clitoral development the following structures are seen: the central clitoral mesenchyme (the homolog of central penile mesenchyme), the lateral and ventral mesenchymal columns, all

Table 1
Comparison of morphology of prenatally oil- and flutamide-treated adult rat penises.

Prenatally oil-treated	Prenatally flutamide-treated
Penile Urethra	**Penile Urethra**
a. Normal	a. Abnormal
b. Distal meatus, normal	b. Proximal meatus, abnormal
c. Dorsal blind-ending extensions = absent	c. Dorsal blind-ending extensions = present
d. Urethra completely within penis	d. Urethra partially within wall of external prepuce
MUMP Cartilage	**MUMP Cartilage**
a. Normal length	a. Reduced length
MUMP Corpora Cavernosa	**MUMP Corpora Cavernosa**
a. Normal	a. Hypoplastic
Internal Prepuce	**Internal Prepuce**
a. Formed from 3 components	a. Formed from 3 components
b. Normal fusion of 3 components resulting in the ventral raphe	b. Failure of mid-ventral fusion
c. Shallow dorsal-lateral raphes denoting fusions	c. Deep dorsal-lateral raphes denoting fusions
d. MUMP and urethral meatus obscured by internal prepuce	d. MUMP and urethral meatus visible
e. Circumferential internal prepuce	e. U-Shaped internal prepuce, deficient ventrally
Urethral flaps	**Urethral flaps**
a. Present	a. Present
Corpora Cavernosa Urethrae	**Corpora Cavernosa Urethrae**
a. Present and normal	a. Absent
Os penis	**Os penis**
a. Present	a. Present
Corpus Cavernosum Glandis	**Corpus Cavernosum Glandis**
a. Present and normal	a. Present and hypoplastic
b. Completely circular	b. Deficient ventrally
Ventral Tethering	**Ventral Tethering**
a. Absent	a. Present
Preputial space	**Preputial space**
a. Circular	a. U-shaped, deficient ventrally
Penis	**Penis**
a. Circular transverse profile	a. U-shaped transverse profile
b. Mobile (untethered)	b. Immobile (tethered)
Furrowed mucosa	**Furrowed mucosa**
a. Present, normal	a. Hypoplastic, abnormal
b. Lightly H & E stained	b. Densely H & E stained

circumscribed by epithelium (compare Fig. 13A & C). Lateral mesenchymal columns are present in Fig. 13F and fuse with the central clitoral mesenchyme (Compare Fig. 13G & I). Subsequently, the mesenchyme of the ventral mesenchymal columns (initially completely circumscribed with epithelium [Fig. 13C]) becomes ventrally confluent with ventral mesenchyme in oil-treated females (Fig. 13F). More proximally the urethral epithelium separates from surrounding epithelia resulting in a “stand alone” female urethra (Fig. 13L). An inverted U-shaped clitoral lamina forms (Fig. 13L) whose mesenchyme is bilaterally confluent ventrally and tethered to ventral mesenchyme (Fig. 13L & O, red double-headed arrows). Formation of the female equivalent of an internal prepuce is abortive even though residual epithelial rudiments can be seen in Fig. 13I & L. Fig. 13O illustrates an essentially fully developed clitoris defined by an inverted U-shaped clitoral epithelial lamina. Note also in Fig. 13O the two “urethras”. The ventral urethra conveys urine from the bladder, and the dorsal “urethra” ends blindly as is the case for the flutamide-treated male (Fig. 13N-0). Finally, the well-defined erectile bodies seen in the developing and adult glans penis do not differentiate within the clitoral mesenchyme. In summary, clitoral development involves the formation of rudiments homologous to those involved in penile development. However, the proximal-distal appearance of clitoral rudiments differs from that seen in penile development and the fate of individual element pairs (ventral and lateral columns as well as the rudiments of the “female internal prepuce”) have very different fates.

3.5. Morphometric analysis of normal and abnormal penile development

Male rats treated prenatally with flutamide and euthanized on postnatal day 0 had clear alterations to the penile anatomy. Length of the glans penis and clitoris length was measured, and on postnatal day 0 the oil-treated male glans penis is on average 875 μm (St. Dev. ± 50 μm, n=3) in length while the oil-treated clitoris is 686 μm (St. Dev. ± 64 μm, n=3) long. Average glans penis length in flutamide-treated males on postnatal day 0 was 693 μm (St. Dev. ± 61 μm, n=3), similar to that of the oil-treated control clitoris. At postnatal day 10 length of the glans penis of prenatally oil-treated rats was on average 1785 μm (St. Dev. ± 68 μm, n=3), length of the oil-treated clitoris was 1057 μm (St. Dev. ± 36 μm, n=3), and glans penis length in flutamide-treated male rats averaged 1307 μm (St. Dev. ± 44 μm, n=3), somewhat between the oil-treated male and female controls.

3.6. Androgen receptor expression during normal and flutamide-induced abnormal penile development

Given that flutamide is an androgen receptor antagonist, the spatial distribution of androgen receptors (AR) has important implications in relation to flutamide-induced penile malformations. Accordingly, we examined AR distribution in genital tubercles of prenatally oil- and flutamide-treated rats on the day of birth and at 10 days postnatal to document expression of this transcription factor at critical time points of androgen-dependent development. For this purpose we focused on two morphogenetic events, namely ventral fusion of the preputial-urethral folds to form (a) the urethra and (b) the ventral aspect of the external prepuce. It is important to realize that the day of birth is only about 24–48 h after the last flutamide injection, when flutamide may still be present.

At the day of birth in oil-treated specimens AR protein was detected in mesenchymal cells associated with the preputial-urethral groove, the zone of incipient epithelial fusion to form the distal aspect of the urethra and to complete the external prepuce (Fig. 15A–B). Additionally, AR protein was detected in the central penile mesenchyme (CPM) circumscribed by the external preputial lamina, especially in mesenchymal condensations. The inner basal cells of the external preputial lamina (immediately adjacent to the CPM) were weakly AR-positive (arrowheads in Fig. 15A–B). In addition, dense AR-positive accumulations of blood vessels (BV) were observed bilateral to the preputial-urethral groove of oil-treated specimens (Fig. 15A–B). In a comparable proximal-distal position, the distribution of AR protein was similar in prenatally flutamide-treated specimens with minor exceptions. In this regard, AR expression was undetectable in the epithelium of the external preputial lamina, and AR-positive blood vessels were not present in prenatally flutamide-treated specimens (Fig. 15C-D). In both the oil- and flutamide-treated specimens the epithelium of the preputial-urethral groove was devoid of AR staining, while the adjacent mesenchymal cells were AR-positive (compare Fig. 15 B & D). The epidermis and associated hair follicles were AR-negative in both treatment groups (Fig. 15).

In a more proximal position in newborn genital tubercles, two zones of incipient epithelial fusion were observed, namely zones involved in (a) the formation of the urethra (red arrows in Fig. 16A) and (b) completion of the ventral aspect of the prepuce (black arrows in Fig. 16A). The mesenchymal cells associated with these two areas of incipient epithelial fusion were intensely AR-positive in oil-treated specimens (Fig. 16A). Epithelial fusion to create the penile urethra in this region is associated with mesenchyme of the ventral columns, which in Fig. 16A have become confluent with the central penile mesenchyme (double-headed black arrows in Fig. 16A). The section in Fig. 16B (flutamide treated) is slightly distal to that of Fig. 16A (oil-treated), and thus ventral mesenchymal columns in Fig. 16B are still surrounded by epithelium. It is notable in the flutamide-treated speci-

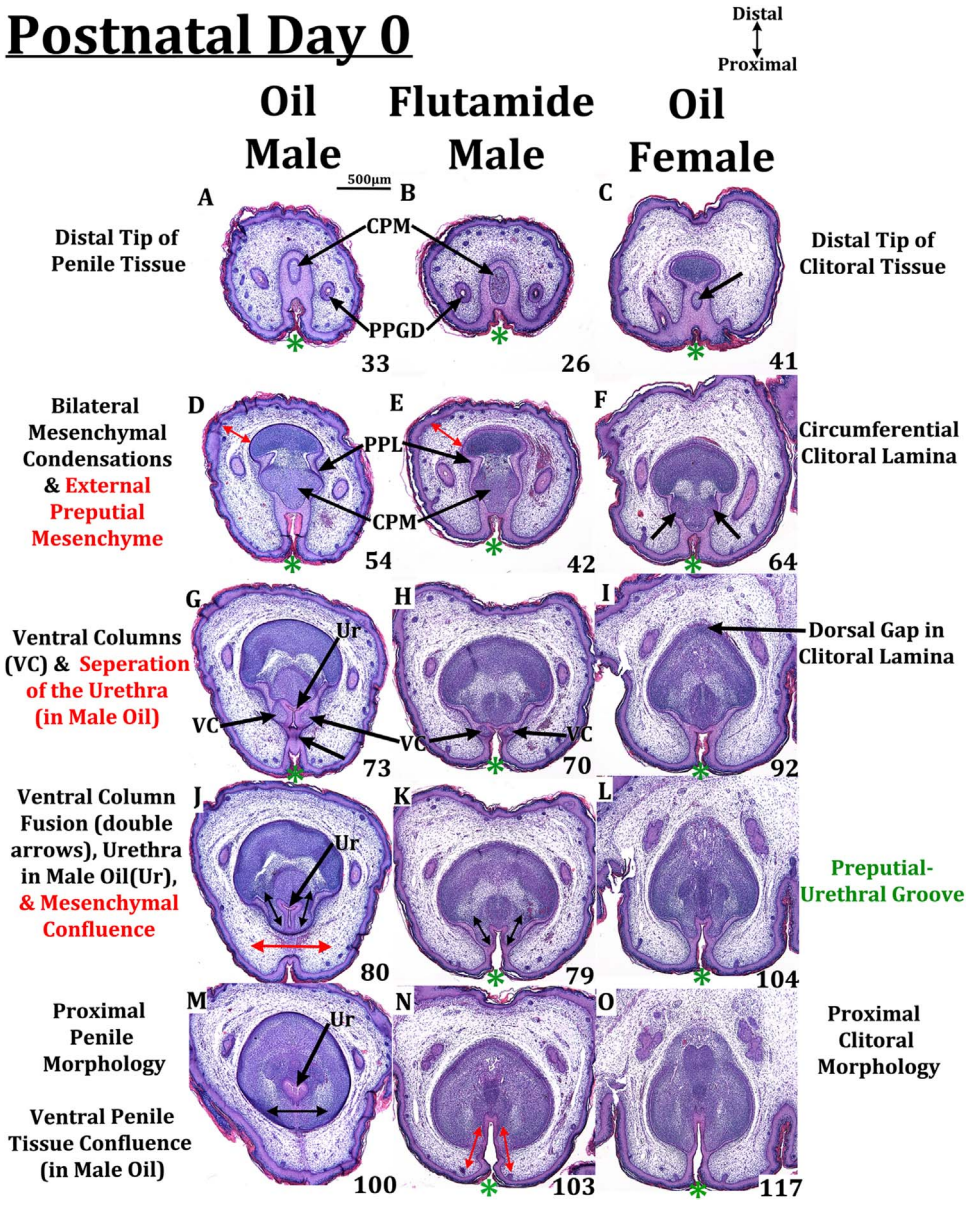

Fig. 12. Transverse sections of the genital tubercle of a postnatal day 0 oil-treated penis stained with hematoxylin and eosin (A, D, G, J, M), prenatally flutamide-treated male (B, E, H, K, N), and prenatally oil-treated female (C, F, I, L, O). The number at the bottom right of each image is the serial section number (section thickness =7 µm). Section numbers start from the distal tip of the external prepuce. Black labels to the right identify the structures referenced by the black arrows in the oil-treated female images. Black labels to the left identify the structures referenced by the black arrows in males; red labels correspond to red arrows in oil- and flutamide-treated male specimens. Green asterisks denote the open preputial-urethral groove. PPGD = preputial gland duct, CPM = central penile mesenchyme, EPL = external preputial lamina, Ur = urethra, VC = ventral columns.

men that mesenchymal AR expression in the ventral mesenchymal columns (VC) is weak (red arrows in Fig. 16B). Likewise, mesenchymal AR expression is more prominent in the zone of incipient epithelial fusion involved in formation of the ventral aspect of the prepuce (apposed black arrows, compare Fig. 16A & B). In oil-treated specimens, AR was also detected in the central penile mesenchyme (especially in mesenchymal condensations), in the inner basal cells of the external preputial lamina (arrowheads), and in mesenchymal cells associated with the external preputial lamina (Fig. 16A). Conversely, in flutamide-treated specimens epithelium of the external preputial lamina was AR-negative, and expression of AR within the central penile mesenchyme was reduced in comparison to that in the oil-treated specimens (Compare Fig. 16A & B).

More proximally in oil-treated newborn genital tubercles, the two fusion events described above have occurred with the formation of a penile urethra completely surrounded with penile mesenchyme and a ventrally complete external prepuce (Fig. 17). AR was strongly expressed in the central penile mesenchyme, including in the zone of mesenchymal confluence immediately ventral to the urethra (double-headed arrow in Fig. 17). Prominent AR expression was also observed in the zone of mesenchymal confluence within the prepuce (seam in Fig. 17). A comparable area does not exist in prenatally flutamide-treated specimens.

AR expression in developing rat penises at 10 days postnatal was also examined to determine whether prenatal flutamide treatment has long-term effects on AR expression. At 10 days postnatal substantial differences in AR localization were observed in developing penises of prenatally oil- and flutamide-treated rats even though flutamide has surely cleared from the animals at this time. For both prenatally oil- and flutamide-treated specimens, AR was broadly detected in epithelial nuclei of the internal and external preputial laminas. In prenatally oil-treated rats penile erectile bodies (corpus cavernosum glandis, MUMP corpora cavernosa, and corpora cavernosa urethrae) were strongly AR-positive, whereas in prenatally flutamide-treated specimens these erectile bodies were poorly

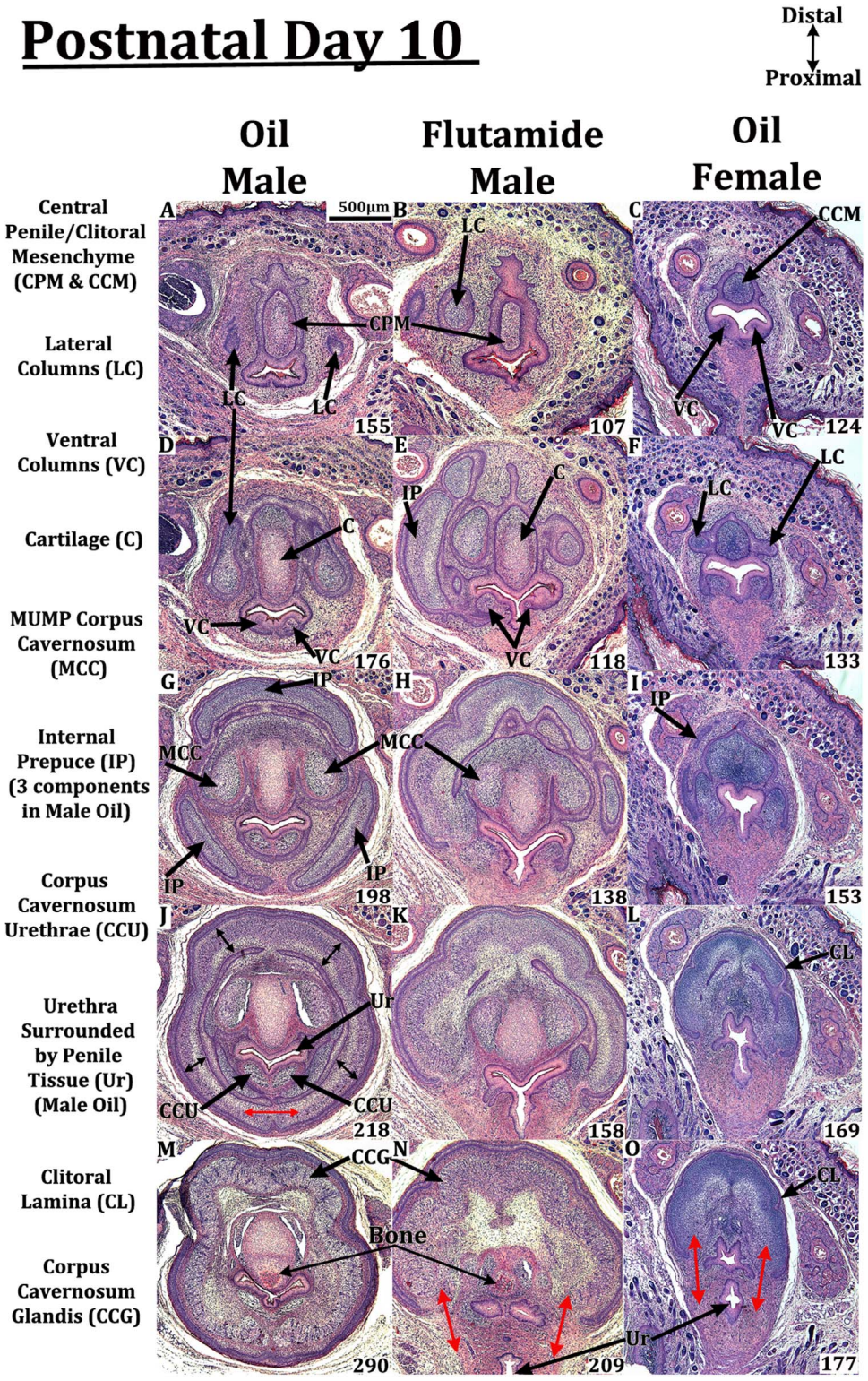

Fig. 13. Transverse sections stained with hematoxylin and eosin of genital tubercles of postnatal day 10 prenatally oil-treated male rats (A, D, G, J, M), prenatally flutamide-treated male rats (B, E, H, K, N), and prenatally oil-treated female rats (C, F, I, L, O). The number at the bottom right of each image is the serial section number (section thickness =7 µm). Section numbers start from the distal tip of the external prepuce. Black labels to the left identify the structures referenced by the black arrows with corresponding abbreviations. In (J) the double-headed black arrows denote the internal prepuce, while the double-headed red arrow denote zones of mesenchymal confluence between adjacent rudiments of the internal prepuce. Red double-headed arrows in N & O denote the confluence of penile/clitoral mesenchyme and ventral mesenchyme. CPM = central penile mesenchyme, CCM = central clitoral mesenchyme, LC = lateral mesenchymal columns, VC = ventral mesenchymal columns, C = cartilage, MCC = MUMP corpora cavernosa, IP = internal prepuce, CCU = corpora cavernosa urethrae, U = urethra, CCG = corpus cavernosum glandis.

developed (or absent in the case of corpora cavernosa urethrae) and reduced in AR expression (Fig. 18A & C). The developing urethras in the two male treatment groups exhibited a vastly different morphology, and the expression of AR in urethral epithelium was different in the two treatment groups (Fig. 18B & D). Deletion of the primary antibody abolished all staining (not illustrated).

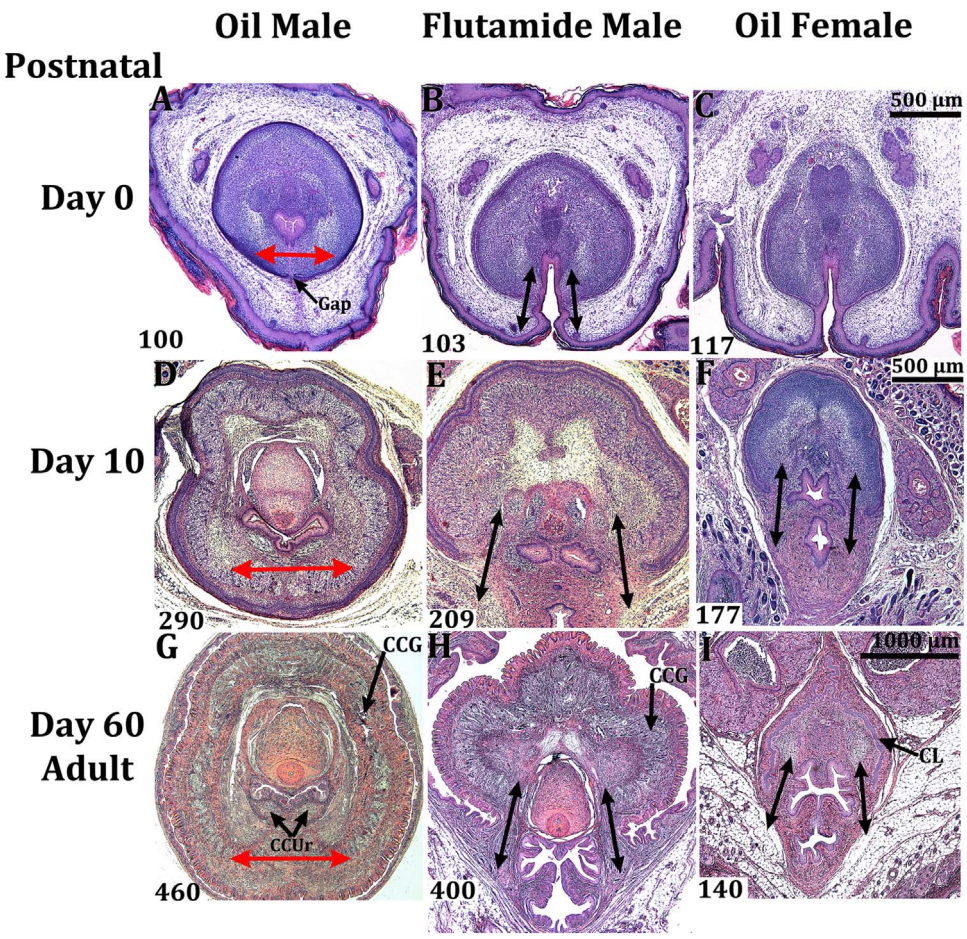

Fig. 14. Transverse sections stained with hematoxylin and eosin of prenatally oil-treated penis at (A) postnatal day 0, (D) day 10, (G) day 60, prenatally flutamide-treated penis at (B) day 0, (E) day 10, (H) day 60, and prenatally oil-treated clitoris at (C) day 0, (F) day 10, (I) day 60. The number at the bottom right of each image is the serial section number (section thickness =7 µm). Black double-headed arrows in flutamide-treated male and oil female specimens denote ventral tethering, the confluence of penile/clitoral tissue and surrounding stroma. Red double-headed arrows in prenatally oil-treated specimens (A, D, G) highlight normal ventral mesenchymal confluence of penile tissue that is absent in prenatally flutamide-treated male and oil-treated female specimens. CCG = corpus cavernosum glandis, and CCUr = corpora cavernosa urethrae, CL = clitoral lamina.

4. Discussion

Flutamide administered prenatally to rats elicits a variety of penile malformations including hypospadias as reported previously (see Introduction). In the current paper, we (a) present for the first time a detailed gross, histologic and SEM description of flutamide-induced rat mid-shaft hypospadias, (b) explore the morphogenetic process of normal rat penile development as well as the developmental events leading to flutamide-induced mid-shaft hypospadias. The current study does not deal with “perineal hypospadias”, the least common form seen in humans. It is unknown whether flutamide at any dose can elicit “perineal hypospadias” in rats. Many of the effects of prenatal flutamide on the rat glans penis can be characterized as partial de-masculinization, recognizing that the malformations are modifications of penile morphology and not complete sex conversion to clitoral morphology. Features indicative of flutamide-induced partial de-masculinization include: (a) extensive clefting of the external prepuce similar to that of females, (b) a blind ending pseudovaginal pouch, (c) reduced size of the external preputial space, (d) reduction of anogenital distance to a value intermediate between normal male and female rats, (e) ventral tethering of the penis, and (f) the blind dorsal urethral extensions and ventral angling of the urethra. In addition, at birth, the distal aspect of the genital tubercle of prenatally flutamide-treated male rats is distinctly female-like having an extensive preputial-urethral groove (Fig. 13). At 10 days postnatal, prenatally flutamide-treated male rats have a U-shaped epithelial lamina that resembles the U-shaped clitoral lamina. The developmental consequences of this U-shaped epithelial lamina in adult prenatally flutamide-treated male rats include: (a) a U-shaped external preputial space (normally circular in males), (b) a U-shaped corpus cavernosum glandis (normally circular in males), (c) bilateral ventral tethering of the penis to the wall of the external prepuce, and (d) a ventrally deficient internal prepuce. Additionally, a trend towards hypoplasia or complete absence of erectile bodies was observed in prenatally flutamide-treated male rats. Distinct erectile bodies are not present in the rat (and mouse) clitoris (Weiss et al., 2012), and adult prenatally flutamide-treated male rats exhibit hypoplastic MUMP corpora cavernosa, a ventrally deficient corpus cavernosum glandis, and complete absence of the corpora cavernosa urethrae. These malformations of the penis are associated with reduction in overall lengths of the glans penis, the MUMP cartilage and the external prepuce, an effect consistent with reduced penile length reported previously in prenatally flutamide-treated male rats at day 25 (Welsh et al., 2008) and in neonatally DES-treated rats (Goyal et al., 2005). Balanced against these feminized features are distinctly masculine (penile) features observed in prenatally flutamide-treated rats (see Table 1) that include an os penis, MUMP, MUMP cartilage, corpus cavernosum glandis, MUMP corpora cavernosa, internal prepuce, urethral flaps, and external preputial space, even though many of these masculine features are malformed.

The first event in sex differentiation of the external genitalia is determination of whether the final outcome will be penis or clitoris (Rodriguez et al., 2012a). Complete sex conversion of external genitalia

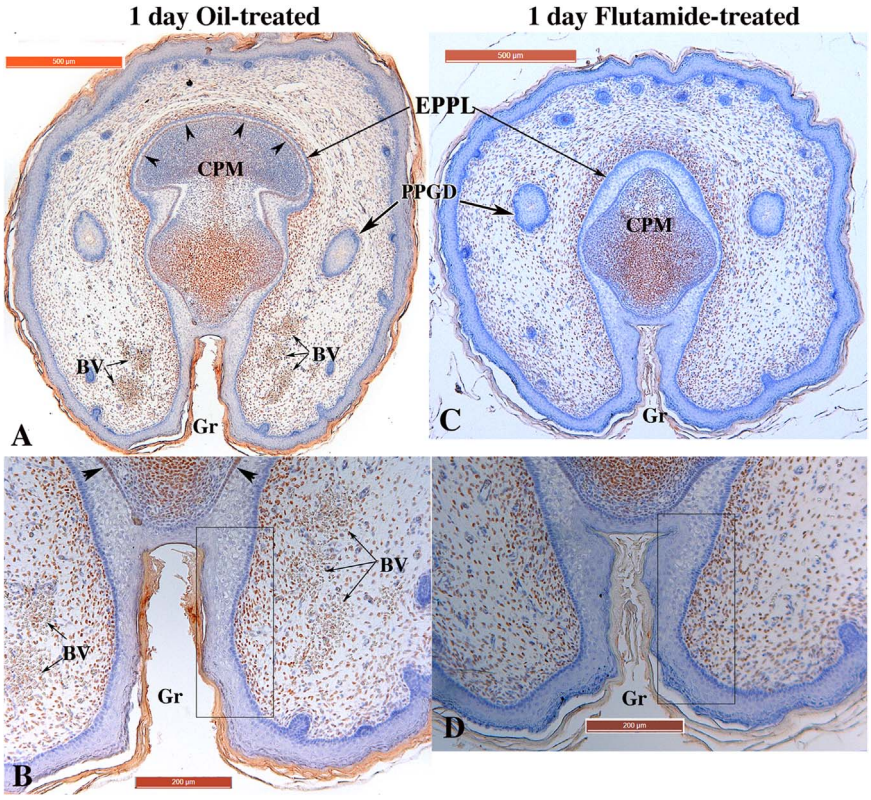

Fig. 15. Genital tubercles of prenatally oil-treated (A & B) and flutamide-treated (C & D) newborn rats stained for androgen receptor. In (A), a distal section, note strong immunostaining of AR in the central penile mesenchyme (CPM) and in blood vessels bilateral to the preputial-urethral groove (Gr). Epithelium of the external preputial lamina exhibits weak AR staining of the inner basal cells (arrowheads in [A & B]), while the epidermis and the epithelium lining the preputial-urethral groove are AR negative. At this comparable proximal-distal level prenatally flutamide-treated specimens exhibit a similar AR protein pattern with the exception of the absence of AR-positive blood vessels (BV). Zones of incipient epithelial fusion in the preputial-urethral groove are virtually identical in the two treatment groups with an AR-negative epithelium and associated AR-positive mesenchymal cells (boxed areas in [B and D]). PPGD = preputial gland duct.

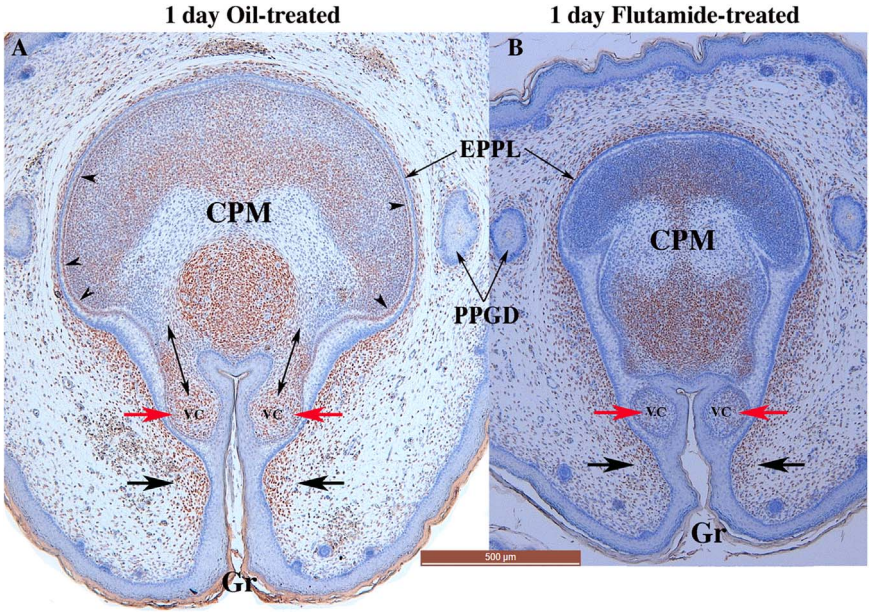

Fig. 16. More proximal sections of genital tubercles of prenatally oil-treated (A & B) and flutamide-treated (C & D) newborn rats stained for androgen receptor near the zone of epithelial fusion and mesenchymal confluence to create a "stand alone" penile urethra (apposed red arrows in [A]) and to complete the prepuce (black arrows in [A]). In the oil-treated specimen (A) the double-headed arrows denote mesenchymal confluence between the ventral mesenchymal columns (strongly AR-positive) and the central penile mesenchyme. Cells of the ventral mesenchymal columns in the flutamide-treated specimen (still surrounded by epithelium) exhibit reduced AR staining. Mesenchymal cells associated with the incipient preputial fusion event (apposed black arrows) are more prominent in the oil-treated versus the flutamide-treated specimen. Note AR-positive basal cells (arrowheads in [A]) in the external preputial lamina (EPPL) in oil-treated, but not in the flutamide-treated specimen. AR staining is more prominent in the central penile mesenchyme (CPM) of the oil-treated versus the flutamide-treated specimen. PPGD = preputial gland duct.

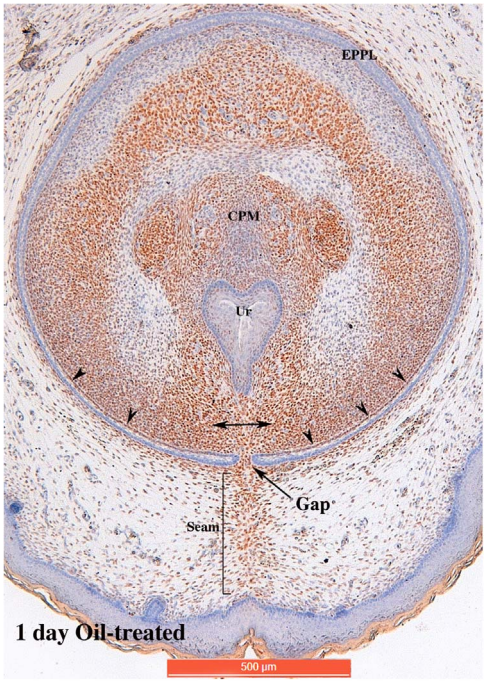

Fig. 17. An even more proximal section of a genital tubercle of a prenatally oil-treated newborn rat stained for androgen receptor. The urethra with its AR-positive epithelium is surrounded by penile mesenchyme (CPM). Note right-left AR-positive mesenchymal confluence ventral to the urethra (double-headed arrow) and the gap in the external preputial lamina (EPPL), whose inner basal cells are AR-positive, especially in ventral areas (arrowheads). The midline zone of mesenchymal confluence (seam) has a density of AR-positive mesenchymal cells.

from masculine (penile) to feminine (clitoral) morphology occurs in X^{Tfm}/Y and androgen receptor null mice, but not as a result of neonatal castration or prenatal flutamide treatment (Rodriguez et al., 2012a; Welsh et al., 2007, 2008, 2010). By all accounts, treatment of rats with flutamide from 14 to 20 days of gestation leads to penile and not clitoral development. Thus, complete sex reversal was not achieved even though a degree of de-masculinization was observed. This means that either the dose of flutamide was not sufficient to achieve complete sex reversal, or that irreversible penile determination occurred prior to initiation of flutamide treatment.

Comparison of the external genitalia within the 3 treatment groups (oil-treated males and females and flutamide-treated males) highlights those developmental processes that are androgen-dependent by virtue of flutamide-induced malformations, including partial de-masculinization described above. Such malformations in the internal and external prepuces, urethra, MUMP and erectile bodies, coupled with the strategic distribution of androgen receptors are indicative of androgen-dependent developmental processes in these structures. That more severe malformations such as complete sex reversal were not seen may be due to inappropriate timing or inadequate dose of flutamide. In this regard, the formation of bone within rat external genitalia is clearly androgen-dependent since an os clitoris (normally absent in female rats) can be induced by neonatal testosterone treatment (Murakami, 1984; Glucksmann and Cherry, 1972). Bone formation (os penis) was not impaired by prenatal flutamide. However, given that the os penis normally forms and differentiates after birth, disruption of bone formation might be achieved following neonatal (as opposed to prenatal) flutamide treatment when the bone rudiment undergoes osteogenesis (Rodriguez et al., 2012a; Murakami, 1987).

Patterning of internal penile elements is androgen-dependent based upon our flutamide experiments. The penis of prenatally oil-treated rats develops a tubular urethra surrounded 360 degrees by penile tissue. In contrast, the urethra of prenatally oil-treated female rats is located partly within the confines of the clitoral epithelial lamina. However, proximally the urethra courses ventral to the clitoral lamina. Thus, the female urethra is never completely confined within the clitoral epithelial lamina. Adult prenatally flutamide-treated male rats exhibit at 10 days a pattern very similar to that of the female having an inverted U-shaped epithelial lamina whose mesenchyme is confluent with ventral mesenchyme, and a urethra similar to that of females in so

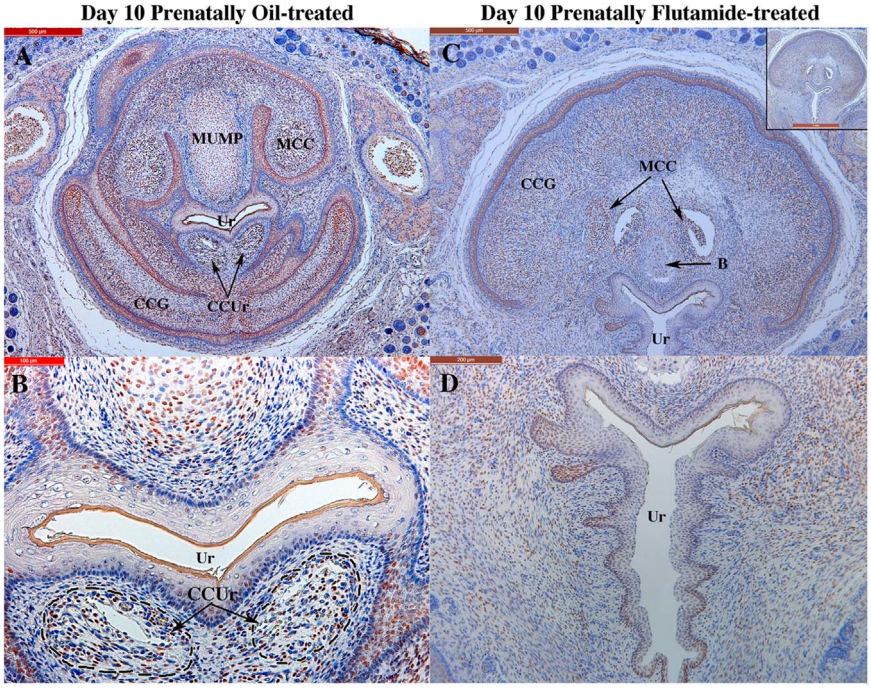

Fig. 18. Sections of developing penises stained for androgen receptor of prenatally oil-treated (A & B) and flutamide-treated (C & D) rats aged to 10 days postnatal. Penile epithelia are strongly stained for AR in both treatment groups (A & C). Erectile bodies are well developed (A) and strongly AR-positive in the oil-treated specimen (corpus cavernosum glandis [CCG], the corpora cavernosa urethrae [CCUr], and MUMP corpora cavernosa [MCC]). In contrast, in the flutamide-treated specimen these erectile bodies are poorly developed and reduced in AR staining (C). Urethral morphology (Ur) and AR expression is different in oil- versus flutamide-treated specimens (B & D). B = bone.

Table 2
Comparison of penile structures in rats and mice.

Structure	Mouse	Reference	Rat	Reference
External prepuce	X	(Sinclair et al., 2016c)	X	(Sinclair et al., 2016c)
MUMP cartilage	X	(Rodriguez et al., 2011)	X	Current paper
Internal prepuce	X	(Blaschko et al., 2013)	X	Current paper (Cunha et al., 2015)
Penile spines	X	(Rodriguez et al., 2011)	X	(Orr and Brennan, 2016)
Os penis	X	(Rodriguez et al., 2011)	X	(Orr and Brennan, 2016)
Urethra	X	(Rodriguez et al., 2011)	X	(MacLellan and Diamond, 2006; Murakami, 1984)
MUMP Corpora cavernosa	X	(Rodriguez et al., 2011)	X	Current paper
Urethral Flaps	X	(Rodriguez et al., 2011)	X	Current paper
Corpora cavernosa urethrae	X	(Rodriguez et al., 2011)	X	Current paper
Corpus Cavernosum Glandis	X	(Rodriguez et al., 2011)	X	(Murakami, 1984)
Furrowed mucosa	NO		YES	Current paper

far as the urethra tracks into mesenchyme ventral to the penis. In adulthood, the penis of prenatally flutamide-treated male rats is ventrally tethered bilaterally, and the urethra is embedded within the ventral wall of the external prepuce instead of being confined solely within the penis.

Definitive and substantial mid-shaft hypospadias was observed in prenatally flutamide-treated male rats and was manifest in adulthood as (a) substantial proximal shifting of the urethral meatus from its normal distal position, (b) ventral tethering of the penis to the external prepuce, (c) ventral defects in the internal and external prepuces and (d) absence of the corpora cavernosa urethrae (the rat/mouse homolog of the human corpus spongiosum). These malformations of rat external genitalia are completely consistent with the definition of mid-shaft hypospadias (Cunha et al., 2015).

4.1. Rat/Mouse comparison

Rat and mouse penile anatomy and development are remarkably similar as indicated in Table 2. For many years the terminology describing the anatomical features of the mouse penis was imprecise and frankly inadequate. Accordingly, our recent studies have addressed this issue (Rodriguez et al., 2011, 2012a; Yang et al., 2010; Blaschko et al., 2013; Mahawong et al., 2014b, 2014a; Sinclair et al., 2016a, 2016b). In the course of our studies, which have been recently confirmed by the work of Phillips (Phillips et al., 2015), we have introduced descriptive terms such as the male urogenital mating protuberance (MUMP), MUMP ridge, MUMP corpora cavernosa, internal and external prepuces. However, as our analysis has proceeded some of the anatomical terms originally proposed have evolved somewhat. The terms used in the current paper supersede previous terminology. Prior to this report it was unknown whether rats have corpora cavernosa urethrae or MUMP corpora cavernosa as is the case for mouse (Rodriguez et al., 2011). It is now apparent that the rat has both of these erectile bodies. One difference in penile anatomy in rats and mice is that the mouse urethral meatus is visible where the MUMP joins the MUMP ridge. In contrast, in rats the urethral meatus is not visible as it is obscured by the furrowed mucosa and internal prepuce. Another major difference between adult rat and mouse penises is that the cartilaginous MUMP is a prominent distal projection of the mouse penis (Rodriguez et al., 2011), whereas the cartilaginous MUMP of the rat is completely covered by the internal prepuce and thus is not visible. A surprising observation is the increase in length of the MUMP cartilage relative to overall length of the glans penis in prenatally oil- versus flutamide-treated rats. However, prenatal flutamide treatment actually decreases absolute length of the MUMP by ~67%, and also decreases the total length of the glans penis by an even larger amount.

Given the similarities in adult penile morphology in rats and mice, it is not surprising that the developmental processes and the rudiments generating erectile bodies and other penile structures are similar/identical in these two species with a few exceptions (Table 3). It is notable, however, that while the internal prepuce is present in both rats and mice, the developmental process is distinctly different in rats and mice. In the case of the rat the internal prepuce develops as a result of fusion of three separate rudiments, a process different than that seen in the mouse (Schlomer et al., 2013; Sinclair et al., 2016a). Fusion of the three separate rudiments of the rat internal prepuce is manifested in adulthood by prominent grooves/raphes not seen in mice. The furrowed mucosa at the tip of the penis is unique to the rat and is not present in mice. While the morphogenesis of this structure in not known, during erection in the rat an elaborate “cup-shaped” structure unfurls from the distal aspect of the penis (Kondo et al., 1997). The highly folded, complex, redundant morphology of the furrowed mucosa at the tip of the rat penis is surely the morphological entity from which the “cup-shaped” structure arises during erection.

4.2. Fusion events in penile development

Formation of the human penile urethra occurs via fusion of the urethral folds to convert the urethral groove into a tubular urethra (Li et al., 2015b; Shen et al., 2016). This process occurs within the genital tubercle, which projects into the amniotic cavity, and thus fusion of the human urethral folds occurs in an amniotic fluid environment. Penile development in rats and mice differs from that of humans in that most of the developmental process occurs deep to the tissues of the external prepuce after the preputial folds have over-grown the genital tubercle during late fetal and neonatal periods (Rodriguez et al., 2011, 2012a; Schlomer et al., 2013; Petiot et al., 2005; Perriton et al., 2002; Baskin et al., 1998). Thus, most penile development, including fusion events, occur postnatally deep to the external body surface and not in contact with amniotic fluid in rats (and mice). This human versus rodent species difference may have critical implications in regard to the proximal-distal position of hormonally induced penile malformations such as hypospadias in rodents. Despite this major species difference, the presence of ventral raphes in the human, rat and mouse penis (Cunha et al., 2015; Clemente, 1985; Rodriguez et al., 2011) are indicative of developmental fusion events in all three species. The prominent ventral penile raphe in rats is particularly striking in that it extends the full length of the rat penis from its distal tip to the glans-body junction. While the distal aspect of the rat penile raphe is located within the internal prepuce, most of the rat penile raphe is proximal to the internal prepuce and therefore must be due to different unique fusion events to be discussed below.

It is important to realize that the surface of the rat and mouse penis (especially the ventral penile surface) is revealed (formed) as a result of delamination of the external preputial epithelial lamina which occurs in mice between 24 and 30 days postnatal (Mahawong et al., 2014b). The timing of delamination of the external preputial epithelial lamina occurs in a similar time frame in rats as this process is known to be

Table 3
Comparison of developmental structures and processes.

Structure	Mouse	Reference	Rat	Reference
Dorsal mesenchymal columns	YES	(Schlomer et al., 2013)	NOT Observed	Current paper
Lateral mesenchymal columns	YES	(Schlomer et al., 2013)	YES	Current paper
Ventral mesenchymal columns	YES	(Schlomer et al., 2013)	YES	Current paper
Central penile mesenchyme	YES	(Schlomer et al., 2013)	YES	Current paper
External preputial folds	YES	(Perriton et al., 2002; Petiot et al., 2005)	YES	(Kluth et al., 2011)
External preputial lamina	YES	(Sinclair et al., 2016a)	YES	Current paper
Internal preputial lamina	YES	(Sinclair et al., 2016a)	YES	Current paper
Three internal preputial rudiments	NO		YES	Current paper
MUMP rudiment	YES	(Rodriguez et al., 2012a)	YES	Current paper
Preputial-urethral groove	YES	(Sinclair et al., 2016a)	YES	Current paper
Ventral gap in external preputial lamina	YES	(Sinclair et al., 2016a)	YES	Current paper
Epithelial fusion events	YES	(Sinclair et al., 2016a)	YES	Current paper
Mesenchymal confluence events	YES	(Sinclair et al., 2016a)	YES	Current paper

associated with the onset of puberty (Korenbrot et al., 1977). From birth to about 24 days postnatal (before delamination of the external preputial lamina), the male mouse external preputial lamina normally has a very narrow ventral gap through which penile stroma is confluent with stroma of the external prepuce (Mahawong et al., 2014b; Rodriguez et al., 2012a; Sinclair et al., 2016a). This gap is also seen in the developing rat penis. In order to reveal (form) the emerging ventral penile surface during delamination of the external preputial lamina, the stromal gap in the external preputial lamina must be sealed as a result of epithelial-epithelial fusion. This ventral epithelial-epithelial fusion requires close apposition of the right and left ventral halves of the external preputial lamina, a process severely impaired by prenatal flutamide treatment, which results in a U-shaped external preputial lamina, broad bilateral stromal confluence between penile and ventral preputial stromas, and extensive ventral tethering of the rat penis in adulthood. Impaired fusion events in penile development also altered the morphology of the distal tip of the penis, impaired development of the furrowed mucosa, proximal shift the urethral meatus, and completely abrogation of development of the corpora cavernosa urethrae normally located immediately ventral to the urethra.

Evidence for fusion events is particularly compelling in regard to formation of the rat internal prepuce, which forms from three rudiments manifested in adulthood as the prominent ventral penile raphe on the internal prepuce as well as lateral grooves on the external surface of the internal prepuce. Fusion of the three rudiments of the developing rat internal prepuce occurs deep to the surrounding external prepuce and involves: (a) bilateral fusion of the right and left ventral rudiments with the dorsal rudiment, and (b) mid-ventral fusion of the right and left ventral rudiments. The three rudiments of the internal prepuce are initially circumscribed by epithelium, and each epithelial element is in turn separated from the others by mesenchymal tissue. To form the circumferential internal prepuce, several events must occur: (a) the mesenchyme separating the 3 rudiments must be displaced to achieve epithelial to epithelial contact and epithelial fusion. (b) Secondarily, the epithelial seams at the points of epithelial contact must be removed to achieve circumferential mesenchymal confluence, thus completing formation of the circular internal prepuce defined by epithelium on its external and internal surfaces with a circumferential core of stromal tissue that differentiates into the corpus cavernosum glandis. These complex developmental events occurring within the internal prepuce are manifest in adulthood by the ventral penile raphe and the bilateral grooves in the 3 and 9 o'clock positions. The fact that the ventral raphe extends proximally to the glans-body junction is indicative of an additional ventral fusion event(s) in more proximal regions of the rat glans penis.

Mesenchymal-mesenchymal fusion events also occur in many places within the developing mouse (Schlomer et al., 2013), rat and human penises (Fig. 19). Many of the rudiments of internal penile structures form as "mesenchymal cores or islands" surrounded by epithelium. In the mouse these are the dorsal, lateral and ventral mesenchymal columns (Schlomer et al., 2013), which for the most part have direct rat counterparts described herein (Table 3). As mentioned above, the rat internal prepuce forms from epithelial fusion of the three separate rudiments, subsequent epithelial seam removal and then mesenchymal confluence. During embryonic development, the external prepuce of rats and mice is also formed by mid-ventral epithelial-epithelial fusion of the preputial folds, that eventually completely cover the penile rudiment (Perriton et al., 2002; Petiot et al., 2005; Baskin et al., 1998; Kluth et al., 2011; Lin et al., 2016). Removal of the midline preputial epithelial seam is followed by midline mesenchymal confluence within the external prepuce (Sinclair et al., 2016a). In mice, another mesenchymal-mesenchymal fusion event occurs during the establishment of mesenchymal confluence between right and left dorsal columns to form the central penile mesenchyme (Schlomer et al., 2013). It is not yet known whether bilateral dorsal mesenchymal columns are present and involved in the development of the central penile mesenchyme of the rat. However, in both rats and mice, mesenchymal confluence also occurs between right and left lateral columns and the central penile mesenchyme and between right and left ventral columns and central penile mesenchyme (Sinclair et al., 2016a, 2016b; Rodriguez et al., 2012a). These highly orchestrated and spatially precise epithelial and mesenchymal fusion events are required to generate a normal, anatomically appropriate and functional penis. Prenatal flutamide impairs many of these epithelial and mesenchymal fusion events leading to an abnormal external prepuce with an exaggerated distal cleft similar to that of females. Likewise, prenatal flutamide elicits formation of an abnormal internal prepuce and an abnormal corpus cavernosum glandis, which are deficient ventrally. Failure of epithelial-epithelial fusion and subsequent mesenchymal confluence also results in an external preputial space that is U-shaped as opposed to its normal circular shape. These prenatally flutamide-induced developmental impairments of epithelial-epithelial fusion and subsequent impaired mesenchymal confluence have global effects on the position and morphology of the rat penile urethra as well as abnormal ventral tethering of the penis to the inner wall of the external prepuce. The morphogenetic and molecular events involved in formation of epithelial seams at the point of fusion, subsequent elimination of epithelial seams and establishment of mesenchymal confluence are not understood, but clearly merit future investigation.

Another example of mesenchymal confluence undoubtedly occurs in formation of the proximal portion of the mouse penile urethra, a process, which may also occur in rats. In mice the proximal portion of the penile urethra forms by direct canalization of the urethral plate (Hynes and Fraher, 2004a; Seifert et al., 2008), which initially is attached to ventral ectoderm (Fig. 19 E-F). As penile urethral development proceeds in embryonic mice, the attachment of the urethral plate to the surface ectoderm is eliminated followed by midline

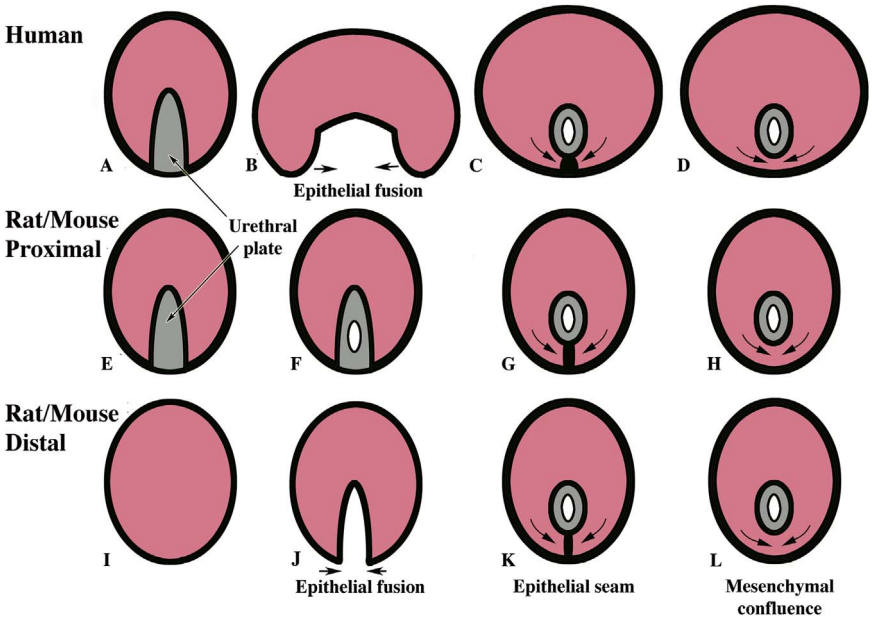

Fig. 19. Diagramatic representations of penile urethral development in human (A–D), mouse proximal (E-H) and mouse/rat distal (I–L). Penile urethral development in human (A–D) and mouse and rat distal (I–L) occur via similar mechanisms: (a) formation of a ventral groove (B & J), (b) epithelial fusion to close the groove and to form an epithelial seam (C & K), (c) epithelial seam removal and mesenchymal confluence (C-D & K-L). Formation of the open urethral groove in humans occurs via canalization of the urethral plate (A–B). Note the absence of a urethral plate in the distal aspect of the mouse genital tubercle (I), and the formation of the preputial-urethral groove (J) (Sinclair et al., 2016a, 2016b). Formation of the proximal urethra in mice involves formation of a urethral plate attached to the surrounding ectoderm (E), direct canalization of the urethral plate to generate a urethral lumen (F), removal of the epithelial seam and ventral mesenchymal confluence (G–H).

mesenchymal fusion (confluence) ventral to the urethra (Fig. 19G–H). In so doing the murine penile urethra becomes a “stand alone” tube completely surrounded by penile mesenchyme (Fig. 19H). During human penile development, fusion of the urethral folds generates as epithelial seam (Fig. 19C), which upon elimination allows for mid-ventral mesenchymal fusion (Fig. 19D), a process that also occurs in the development of the distal aspect of the mouse penile urethra (Fig. 19J–L). These midline epithelial and mesenchymal fusion events are likely responsible for the ventral penile raphe seen in adult rats, mice and humans. A similar process occurs in development of the proximal aspect of the rat penile urethra (Kluth et al., 2011), and appears to account for the proximal portion of the rat ventral penile raphe seen in adulthood. While penile developmental processes differ substantially in humans versus rats and mice (Fig. 19), there are common developmental processes, which are basically the same in all three species: (a) epithelial-epithelial contact and fusion, (b) elimination of epithelial seams and (c) subsequent mesenchymal confluence (Fig. 19).

4.3. Androgen receptor expression

In this study pregnant rats were treated with flutamide from day 14–20 of gestation. Given that certain effects of flutamide were seen at birth (one day after the last flutamide injection), it is appropriate to assess the normal pattern of AR expression at this stage. At birth prominent mesenchymal expression of AR was seen throughout the central penile mesenchyme (especially in mesenchymal condensations) and within mesenchymal cells in intimate association with the external preputial lamina in prenatally oil-treated rats. Similar patterns of AR expression have been documented in the developing mouse penis (Blaschko et al., 2013; Rodriguez et al., 2012a). AR-positive cells within penile mesenchymal condensations are the precursors that differentiate into AR-positive erectile bodies (MUMP corpora cavernosa, corpora cavernosa urethrae, and corpus cavernosum glandis) observed at day 10. Prenatal flutamide treatment impaired development of penile erectile bodies concomitant with reduction in AR expression. Thus, androgens are likely to be required in differentiation of erectile bodies from mesenchymal precursors. This interpretation is supported by the findings in prenatally flutamide-treated rats of the ventral defect in the corpus cavernosum glandis and the complete absence of the corpora cavernosa urethrae. Finally, AR was strategically expressed in mesenchymal cells associated with zones of incipient epithelial fusion and mesenchymal confluence, which is consistent with the flutamide-induced fusion defects resulting in (a) ventrally deficient internal prepuce, (b) ventrally deficient corpus cavernosum glandis, (c) ventrally deficient external preputial space and (d) ventral tethering.

Hormonal regulation of AR is complicated and may vary substantially in specific cell/tissue types. Accordingly, up-regulation of AR by testosterone has been reported (Block et al., 1991; Ma et al., 2005; Ubels et al., 2003; Bentvelsen et al., 1994). Likewise, AR is down-regulated by flutamide (Durlej et al., 2012; Kontula et al., 1985). Consistent with these reports, we observed several instances of flutamide-induced reduction in AR protein levels in the developing rat penis. The epithelium of the external preputial lamina prenatally oil-treated newborn rats exhibited distinct AR expression. In newborn specimens, this epithelial expression of AR was abolished by prenatal treatment with flutamide. By 10 days postnatal, the weak epithelial AR immunostaining in oil-treated specimens up-regulates globally to strong AR expression within most internal penile epithelia. Likewise, the uniformly AR-negative epithelia within the prenatally flutamide-treated specimens seen at birth becomes strongly AR-positive by day 10. Thus, there is a distinct ontogeny of epithelial AR protein immunostaining in male external genitalia, and the complete absence of epithelial AR staining seen at birth in prenatally flutamide-treated specimens, recovers following discontinuation of flutamide treatment, implying that the absence of AR immunostaining seen at birth is reversible.

In prenatally flutamide-treated rats mesenchymal AR expression was dramatically reduced at birth in the central penile mesenchyme and the ventral mesenchymal columns. Comparison of prenatally oil- versus flutamide-treated specimens at day 10 reveals a continued (perhaps irreversible) reduction in AR expression. Hormonal treatments (estrogen or flutamide) during perinatal periods are known to lead to reduction in AR expression in adult reproductive tract

structures (Durlej et al., 2012; Prins, 1992; Prins and Birch, 1995; Woodham et al., 2003).

The preputial-urethral groove undergoes two epithelial fusion events followed by mesenchymal confluence to generate a “stand alone” penile urethra and to complete the ventral aspect of the prepuce in both mice (Sinclair et al., 2016a) and rats (current paper). Epithelial AR in the preputial-urethral groove was undetectable in the region of right-left preputial fusion, while the associated mesenchymal cells were AR-positive. Epithelial shape changes leading to fusion are clearly androgen-dependent (inhibited by prenatal flutamide and absent in females). Accordingly, effects of androgens (and anti-androgens such as flutamide) on epithelial morphogenesis during this morphogenetic process appear to be due to paracrine mechanisms mediated through mesenchymal AR as demonstrated previously for prostatic development (Cunha and Lung, 1978).

In summary, the current paper provides the first detailed description of prenatally flutamide-induced mid-shaft hypospadias based upon gross, histologic, morphometric and SEM observations. Similar malformations are likely in rats treated with other agents that either block androgen action at the site of the androgen receptor or the production of androgens by the testes. The status of penile defects elicited by estrogens or 5α-reductase inhibitors in rats remains to be determined, but given the interplay between androgen and estrogen signaling in development of external genitalia (Zheng et al., 2015), a detailed gross, histologic and SEM examination of estrogen-induced rat penile malformations (hypospadias) is imperative. The types of adult penile malformations elicited by prenatal flutamide are consistent with perturbation of processes involved in normal penile development and with the tissue distribution of androgen receptors. Taken together, the current study emphasizes the utility of the rat as a model for human mid-shaft hypospadias.

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